



Exploring the Role of Ubiquitylation in the Mammalian DNA Damage Response

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Publication date:
2009

Document Version
Publisher's PDF, also known as Version of record

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Citation (APA):
Kildegaard, H. F. (2009). *Exploring the Role of Ubiquitylation in the Mammalian DNA Damage Response*. Technical University of Denmark.

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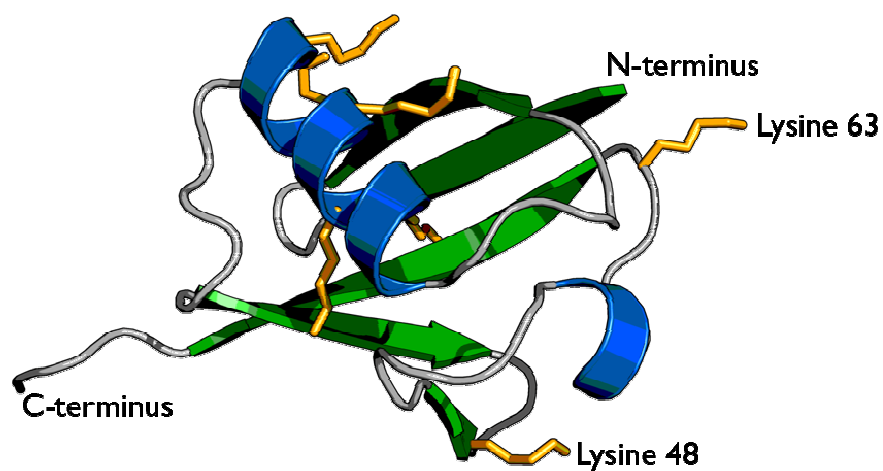
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Exploring the Role of Ubiquitylation in the Mammalian DNA Damage Response

PhD thesis by
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November 2008

Publications

Publication 1: Niels Mailand, Simon Bekker-Jensen, **Helene Faustrup**, Fredrik Melander, Jiri Bartek, Claudia Lukas and Jiri Lukas. RNF8 Ubiquitylates Histones at DNA Double-Strand Breaks and Promotes Assembly of Repair Proteins. Cell 131, 887-900, 2007.

Publication 2: **Helene Faustrup**, Simon Bekker-Jensen, Jiri Bartek, Jiri Lukas and Niels Mailand. USP7 counteracts SCF ^{β TrCP}- but not APC^{Cdh1}-mediated proteolysis of Claspin. Journal of Cell Biology 184, 13-19, 2009.

About the cover:

The picture is a cartoon representation of ubiquitin highlighting the secondary structure. α -helices are coloured in blue and β -strands in green. The side chains of the 7 lysine residues applied for ubiquitin chain formation are indicated by orange sticks and the two most applied K48 and K63 are labeled. Image name: Ubiquitin cartoon-2-.png which is downloaded from Wikimedia Commons.

Preface

This PhD has been carried out at the Centre for Genotoxic Stress Research at the Danish Cancer Society (DCS) in the lab of Dr. Jiri Lukas and Dr. Jiri Bartek. I am very grateful to Jiri Lukas and Jiri Bartek for giving me this unique opportunity to do research in one of the best labs in Denmark. Especially, I would like to thank Jiri Lukas for supervising me, for his great suggestions, ideas and support.

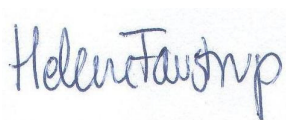
During the PhD, I have been enrolled at the Technical University of Denmark (DTU) as a PhD student in the group of associate professor Uffe Hasbro Mortensen, at the Center for Microbial Biotechnology. I would like to thank Uffe for accepting me as a PhD student and for giving me the opportunity to present my work in his group. I am very grateful for his supervision, good advices and for helping me with administrative issues at DTU.

This PhD was financed by the Integrated Project on DNA Repair, which is part of the 6th Framework Program of the European Union. I am very thankful to have been involved in this project due to the exciting research and possibility of networking with other young scientist around Europe. Furthermore, I had the great opportunity to participate in courses and meetings, organized by participants in the EU project, in Holland, Belgium, Italy and Portugal.

I am very grateful for having the chance of doing research together with especially three very skilled colleagues; Niels Mailand, Simon Bekker-Jensen and Claudia Lukas. Niels have been supervising me in the lab the last two years and I am very thankful for his knowledge, skills, suggestions and ideas. For this, I also thank Simon, who I have known for nine and a half year, since we started at DTU. I am also grateful to Simon for his support and for introducing me to Jiri Lukas and Uffe Mortensen.

The last three years have been very exciting, but also challenging, and I am very thankful to all my colleagues at both DCS and DTU for their support. Especially Dorthe, Carsten, Petra, Simon, Myung Hee, Niels and Jannie have meant a great deal to me, and I will leave DCS with many good memories. Also, I owe a great deal to my family and friends, who have taken care of me, supported me and encouraged me for all these years of education. Finally, I am very thankful to my better half, Jonas, for his care, support and patience.

Copenhagen, November 28, 2008



Helene Faustrup

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1 Abstract

Since the discovery of the protein modifier ubiquitin, the knowledge and understanding of its role in cellular processes has increased tremendously. In the area of DNA damage response (DDR), ubiquitylation has been shown to have important roles in facilitating checkpoint signaling and DNA repair upon exposure to genotoxic stress. Many ubiquitylated proteins, ubiquitin ligases and deubiquitylating enzymes with roles in the DDR pathways have been discovered.

The genomes of all living species, including mammalian cells, are under constant attack by DNA damaging agents from both endogenous and exogenous sources. To restore genomic integrity, damaged DNA needs to be repaired, which require accumulation of repair proteins to the sites of damage. We and others have discovered a role of the ubiquitin ligase RNF8 in DDR, which has increased our knowledge on the mechanism of accumulation of repair proteins to site of DNA damage. We show that RNF8 rapidly assembles at chromatin flanking DNA double-strand breaks (DSBs) via interaction with the mediator protein Mdc1. The assembly of RNF8, which is dependent on phosphorylated Mdc1, is accompanied by an increase in ubiquitin conjugates and accumulation of 53BP1 and BRCA1 at the DSB-flanking chromatin. Disruption of the binding of RNF8 to Mdc1 or its ubiquitin ligase activity impaired the DSB-associated ubiquitylations and inhibited the accumulation of 53BP1 and BRCA1 at the DSB sites. We found the target of RNF8 to be histones H2A and H2AX, and their RNF8 mediated ubiquitylation is suggested to recruit 53BP1 and BRCA1 to the DSB sites, to facilitate repair of the DNA.

Upon DNA damage, DNA repair is coordinated with cell cycle progression through checkpoint mechanisms, which allow cells to arrest the cell cycle until the damage has been repaired. Claspin is an important mediator protein, which facilitates the ATR-mediated phosphorylation and activation of the key checkpoint kinase Chk1. We identified the deubiquitylating enzyme USP7 as a novel regulator of Claspin stability, and USP7 is required to maintain steady-state levels of Claspin. At G2/M, Claspin is degraded by the SCF^{βTrCP} ubiquitin ligase, and over-expression of USP7 was found to counteract this degradation and stabilize Claspin in mitosis. Over-expression of USP7 also increased the half-life of Claspin, which in turn increased the magnitude and duration of Chk1 phosphorylation in response to genotoxic stress, and USP7 was found to deubiquitylate Claspin both *in vivo* and *in vitro*. We furthermore identified Claspin as a substrate of APC^{Cdh1}-mediated degradation in G1, and USP7 was found to only counteract the SCF^{βTrCP}- and not the APC^{Cdh1}-mediated degradation of Claspin.

This thesis has contributed to the discovery of novel roles of ubiquitylation and deubiquitylation in the DDR pathways. Identification of RNF8 has contributed significantly to the understanding of the accumulation of 53BP1 and BRCA1 to the site of damage and the role of ubiquitylation in these processes. Furthermore, the discovery of the role of USP7 in regulating the steady-state level of Claspin has increased the knowledge on the flexible mechanisms to keep a tight regulation of Chk1. There are still many questions to be answered about their exact mechanism and regulation, and it is very likely that ubiquitylation has more roles in the DDR pathways, and more substrates and regulators will be discovered in the future.

2 Dansk Resumé

Siden ubiquitins rolle som postrationel protein modification blev opdaget, er kendskabet og forståelse for dens rolle i cellulære processer vokset kolossalt. Ubiquitylering har vist sig at spille en vigtig rolle i det cellulære respons til DNA-skade, specielt i forbindelse med checkpoint signalering og DNA-reparation efter genotoksisk stress. I de seneste år er mange ubiquitylerede proteiner, ubiquitin ligaser og deubiquitylerings enzymer, med betydning for det cellulære respons til DNA skade, blevet beskrevet.

Genomer i alle levende arter, herunder celler fra pattedyr, er under konstant angreb fra DNA skadelige stoffer af endogen og eksogen art. For at genoprette genomisk integritet skal beskadiget DNA repareres, hvilket kræver en akkumulering af reparationsproteiner omkring DNA skaden. Vi og andre har beskrevet ubiquitin ligasen RNF8's rolle i DNA skade respons, hvilket har øget vores forståelse for de mekanismer, der fører til akkumulering af reparationsproteiner omkring skadet DNA. Vi viser, at RNF8 hurtigt interagerer med chromatin området omkring DNA dobbelt-strengs brud (DSB) via interaktion med adaptor proteinet Mdc1. Akkumulering af RNF8, som er afhængig af fosforyleret Mdc1, er fulgt af en stigning i ubiquitin konjugater og en akkumulering af 53BP1 og BRCA1 omkring det dobbelt-strengede brud. Forstyrrelse af bindingen af RNF8 til Mdc1 eller dets ubiquitin ligase aktivitet nedsatte DSB-associeret ubiquitylering og hæmmede akkumulering af 53BP1 og BRCA1 til DSB. Den direkte funktion af RNF8 formodes at være via ubiquitylering af H2A og H2AX, hvilket rekrutterer 53BP1 og BRCA1 til DNA skaden og muliggør reparation af brudet.

Efter DNA-skade er DNA-reparation koordineret med cellecyklus progression gennem checkpoint mekanismer, der gør det muligt for cellerne at stoppe cellecyklus indtil skaden er blevet repareret. Claspin er et vigtigt protein involveret i ATR-medieret fosforylering og aktivering af den centrale checkpoint kinase Chk1. Vi identificerede det deubiquitylerende enzym USP7 som en ny regulator af Claspin stabilitet, og USP7 er nødvendig for at opretholde steady-state niveau af Claspin. I G2/M nedbrydes Claspin efter ubiquitylering af ubiquitin ligasen, SCF^{βTrCP}, og over-udtryk af USP7 modvirker denne nedbrydning og stabiliserer Claspin i mitose. Over-udtryk af USP7 øger også halveringstiden af Claspin, som igen øger mængden og varigheden af Chk1 phosphorylering efter genotoksisk stress, og USP7 deubiquitylerer Claspin både *in vivo* og *in vitro*. Vi har desuden identificeret Claspin som et mål af APC^{Cdh1}-medieret nedbrydning i G1. USP7 modvirker kun den SCF^{βTrCP}- og ikke den APC^{Cdh1}-medierede nedbrydning af Claspin.

Denne afhandling har bidraget til opdagelsen af nye roller for ubiquitylering og deubiquitylering inden for det cellulære respons til DNA skade. Identifikation af RNF8 har bidraget væsentligt til forståelsen af akkumulering af 53BP1 og BRCA1 omkring steder med DNA skade, og betydningen af ubiquitylering i disse processer. Hertil kommer, at opdagelsen af USP7's rolle i regulering af steady-state niveauet af Claspin har øget vores viden om de fleksible mekanismer, der anvendes til at fastholde en tæt regulering af Chk1. Der er stadig mange spørgsmål, der skal besvares om den nøjagtige mekanisme og dens regulering, og det er meget sandsynligt, at ubiquitylering har flere roller i det cellulære respons til DNA skade, og at flere substrater og proteiner involveret i dets regulering, vil blive opdaget i fremtiden.

3 Introduction

The maintenance of genome integrity is essential for the proper function and survival of all living species. In order to deal with genotoxic insults, mammalian cells have developed elegant mechanisms that detect and repair lesions in the genome. The DNA damage response (DDR) is a multifaceted response that senses DNA damage or replication stress and coordinates the repair with cell cycle progression. The response acts through sensors, transducers and effectors to coordinate cell cycle arrest with DNA repair, cellular senescence and apoptosis, if the damage is too severe. This ensures a low occurrence of mutations, and aberrations in the DDR may therefore lead to developmental defects or genetic diseases including neurodegenerative syndromes, premature aging and cancer (Kastan and Bartek, 2004; Nyberg et al., 2002).

In the last decade, advances have been made in the discovery and understanding of the many proteins and signaling pathways in the DDR. From only knowing the involvement of ATM, p53, p21 in the apoptosis and cell cycle arrest pathways 10 years ago, many more factors and regulating mechanisms have been discovered. One of most important advances is the increased understanding of the involvement of protein phosphorylation and ubiquitylation in executing the cellular responses to DNA damage (Harper and Elledge, 2007).

Ubiquitin was originally isolated in 1975 by Gideon Goldstein and coworkers as the protein ubiquitous immunopoietic polypeptide (UBIP) in a search for hormones of the thymus (Goldstein et al., 1975). Ubiquitin has later been found to be involved in many cellular processes. One of the first links between DDR and ubiquitin was in 1990, where it was shown that the E6 protein from human papillomavirus (HPV) and its associated protein E6-AP were able to bind p53 and promote degradation of p53 via the ubiquitin pathway (Huibregtse et al., 1991; Scheffner et al., 1990). In 1996 it was discovered, that the budding yeast protein Rad6 was an E2 conjugating enzyme and Rad23 contained an ubiquitin binding domain which was required for its function. Both proteins were identified many years earlier in a screen of radiosensitive mutants (Game and Mortimer, 1974). In 2000, Mailand and coworkers discovered ubiquitin- and proteasome- dependent degradation of Cdc25A upon DNA damage (Mailand et al., 2000) and it was not until 2002 that ubiquitylation of PCNA, the link to Rad6, and its involvement in DNA repair was discovered (Hoege et al., 2002).

Today, we know that ubiquitylation has many more substrates and functions in DDR, and ubiquitin has been found to be involved in both protein turnovers, in mediating protein interactions and in regulating protein functions. Indeed, many ubiquitylated proteins, ubiquitin ligases, deubiquitylating enzymes and ubiquitin binding proteins have a role in DDR. And there is still much to learn; there are many uncharacterized proteins with role in ubiquitylation and especially very few of the deubiquitylating enzymes are characterized. Furthermore, when it comes to a specific understanding of the ubiquitin function and regulation, there are still many unanswered questions and mechanisms waiting to be discovered.

The goal of this PhD thesis is to contribute to this knowledge. With the discoveries presented, I hope to shed more light on several mechanism of ubiquitylation and deubiquitylation in the DDR and checkpoint pathways.

The thesis is divided into two main parts which are the “Introduction” and the “Results and Discussion”. In the introduction, the first area described is ubiquitylation which is the main focus of the thesis. In this part, the nature of ubiquitin, mechanism of ubiquitylation, proteasomal degradation, and the established knowledge on ubiquitin ligase and deubiquitylating enzymes (DUBs) are

described. Furthermore, examples of ubiquitylated proteins, ubiquitin ligases and deubiquitylating enzymes involved in DDR are presented. The second area covers the cell cycle and describes what drives the cell cycle and how it is regulated by phosphorylation and ubiquitylation. Focus is put on the different ubiquitin ligases involved in cell cycle regulation. The last area described deals with the cellular responses to DNA damage. This part covers DNA damage, repair pathways and focus is put on the DNA damage response pathways initiated from double strand breaks, with an emphasis on initiation, termination and recovery from the G2/M checkpoint.

In the results and discussion part, the two main projects, I have been involved in, will be presented. In the first project, the novel involvements of the RNF8 ubiquitin ligase in histone ubiquitylation and in facilitating recruitment of 53BP1 and BRCA1 to sites of damage are presented. In the second project, the novel role of the deubiquitylating enzyme USP7 in maintaining steady-state levels of Claspin is presented.

3.1 The Ubiquitin system

Ubiquitylation is a post-translational modification of proteins. Due to the nature of ubiquitin, which is a highly conserved small globular protein of 76 amino acids and 8.5 kDa (Wilkinson and Audhya, 1981), this protein modifier shows greater versatility, than many other types of protein modifications. An extensive amount of proteins are found to be modified by ubiquitin or involved in adding or removing the modifier from proteins. Furthermore, many proteins are found to contain special domains that mediate their binding to ubiquitylated proteins.

Ubiquitin is present in all eukaryotic cells (from yeast to humans) and is present in all cell compartments, where it is involved in both selective degradations of many short-lived (also some long-lived) proteins, and in changing the properties and localization of many proteins. The ubiquitin system is involved in many cellular processes including intracellular trafficking, signal transduction, cell cycle progression, transcriptional regulation, receptor down-regulation, endocytosis, immune response, apoptosis and DNA repair (Haglund and Dikic, 2005; Hershko and Ciechanover, 1998; Tanaka and Chiba, 1998).

Ubiquitylation

Ubiquitin becomes covalently attached to the lysine residues (N-terminal amino group is also known to be ubiquitylated) of substrate proteins by the action of three classes of enzymes. The first is an ubiquitin activating enzyme (E1). In this step, the C-terminal glycine residue (Gly76) of ubiquitin is activated in an ATP requiring step, and ubiquitin is bound to a cysteine residue of the E1 enzyme. In the second step, the activated ubiquitin is transferred to a cysteine residue of an ubiquitin conjugating enzyme (E2). Third, an ubiquitin ligating enzyme (E3, ubiquitin ligase) links the C-terminus of ubiquitin to the ϵ -amino group of a lysine residue in the substrate protein, through formation of an isopeptide bond (Hershko et al., 1983; Wilkinson, 1995). An overview of the ubiquitylation reaction is given in Figure 1 and a representation of ubiquitin is given in Figure 2A.

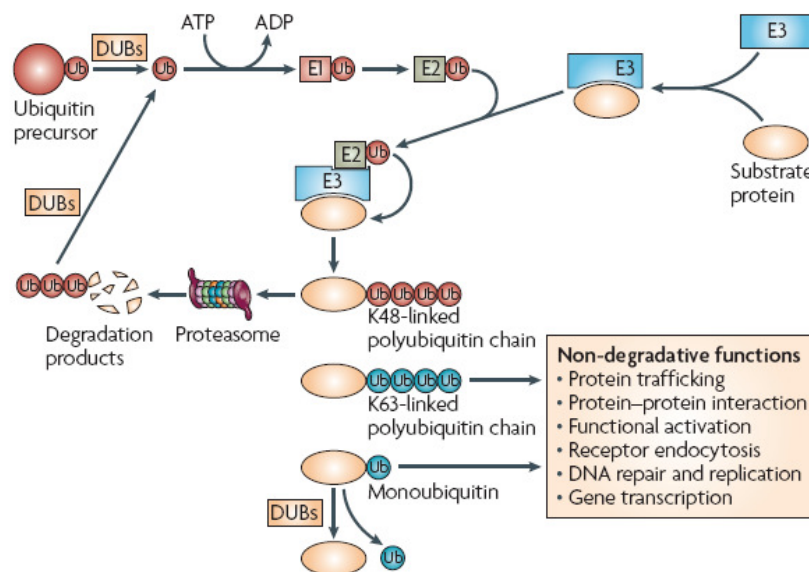


Figure 1: An overview of protein ubiquitylation. Ubiquitylation involves conjugation of one or more ubiquitin moieties onto a substrate protein by the action of an ubiquitin activating enzyme (E1), an ubiquitin conjugating enzyme (E2) and an ubiquitin ligating enzyme (E3). Ubiquitylated proteins can be degraded in the proteasome or have non-degradative functions. Deubiquitylating enzymes (DUBs) prepare free ubiquitin from precursors or remove ubiquitin from ubiquitin chains or directly from substrates (Sun, 2008).

The covalent attachment of ubiquitin to substrate proteins can result in monoubiquitylation, where a single ubiquitin is conjugated to the protein. If the protein is multiubiquitylated, several ubiquitin moieties are linked to the same protein, but each at a different site. Furthermore, proteins can become polyubiquitylated, where several ubiquitin moieties are linked through isopeptide bonds between the c-terminal glycine in one ubiquitin to a lysine in another ubiquitin, forming an ubiquitin polymer. See Figure 2C. Ubiquitin contains seven different lysines which are: K6, K11, K27, K29, K33, K48 and K63 and the five most common lysines applied in chain formation are presented in Figure 2B. It is believed that all lysines can be used for polyubiquitylation *in vivo* as all were identified in a proteomic analysis of ubiquitin conjugated proteins in yeast (Peng et al., 2003). In addition, the formation of forked polyubiquitin chains have been observed in yeast (Peng et al., 2003) and heterogeneous polyubiquitin chains have been observed *in vitro* (Kim et al., 2007b).

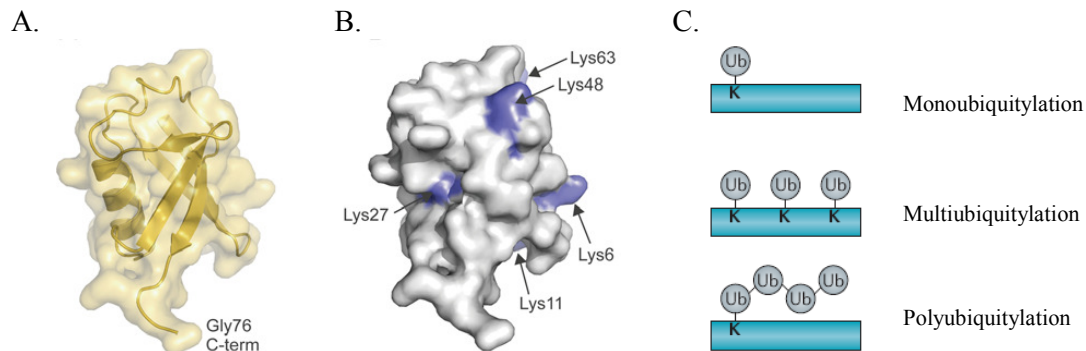


Figure 2: The topology of ubiquitin and examples of ubiquitylation products. (A) Ribbon and surface representation of ubiquitin with the C-terminal Gly76 marked (Hurley et al., 2006). (B) Surface representation of ubiquitin with the five most applied lysines depicted in blue (Hurley et al., 2006). (C) Different types of ubiquitylation products which are monoubiquitylation, multiubiquitylation and polyubiquitylation (Hoeller et al., 2006).

The best characterized lysines of ubiquitin applied for polyubiquitin chains are K48 and K63. In general are K48 linked polyubiquitylated proteins targets of proteasomal degradation, whereas K63 linked polyubiquitylation and monoubiquitylation have more regulatory roles in cellular function. Examples of regulatory roles are to mediate interactions between ubiquitylated proteins and ubiquitin binding proteins, change of protein function and activity and change of cellular localization.

Examples of ubiquitylated proteins involved in DNA damage response

Examples of monoubiquitylated proteins include PCNA, FANCD2, histone H2A, and the TLS polymerases pol η and pol ι . Examples of polyubiquitylated proteins include PCNA, Cdc25A, Claspin, Ku80, DDB2, XPC and RNA polymerase II (Busino et al., 2003; Huang and D'Andrea, 2006; Jason et al., 2002; Mailand et al., 2006; Postow et al., 2008).

Proteasomal degradation

Polyubiquitylated proteins destined for proteolysis are degraded by the 26S proteasome which is present in the cytosol and nucleus of eukaryotic cells (Palmer et al., 1994; Reits et al., 1997; Tanaka and Chiba, 1998). Proteasomal degradation seems to be the major system for selective protein degradation in eukaryotic cells (Hersko and Ciechanover, 1992). The 26S proteasome is a large multisubunit protease complex of approximately 2 MDa and consist of a 20S catalytic core onto which two 19S regulatory subunits are attached (Koster et al., 1995). Ubiquitylated proteins are recognized and bound by the 19S subunits which deubiquitylate, unfold and translocate the proteins into the 20S catalytic core in an ATP-dependent manner, where it is cleaved into short polypeptides (Zwickl et al., 2000). Upon degradation of proteins in the 26S proteasome, free and reusable ubiquitins are released (Ciechanover, 1994). Proteasomal degradation of regulatory proteins is

important in regulating many processes including cell cycle progression, signal transduction, transcription regulation, receptor down-regulation and endocytosis (Hershko and Ciechanover, 1998).

Ubiquitin ligases

Only a few E1 enzymes are known in mammalian cells which are estimated to have more than 20 different E2s and between 500 and 1000 different E3s. Yeast in contrast has only 1 E1, 11 E2 and 54 E3 (Hicke et al., 2005; Hoeller et al., 2006). The main function of E1s and E2s is to prepare ubiquitin for conjugation and E3s are primarily responsible for the specific recognition of target proteins. Ubiquitin ligases exist as single polypeptides or associated with other proteins in multisubunit complexes and are categorized into four major classes based on their specific structural motif. These are the HECT-type, RING-type, U-box-type and PHD-type. Deregulation of several RING-type and HECT-type E3s contributes to cellular tumorigenesis and due to their tumor suppressors or oncogenic properties, they are considered as important potential targets in cancer therapy (Bernassola et al., 2008; Sun, 2003).

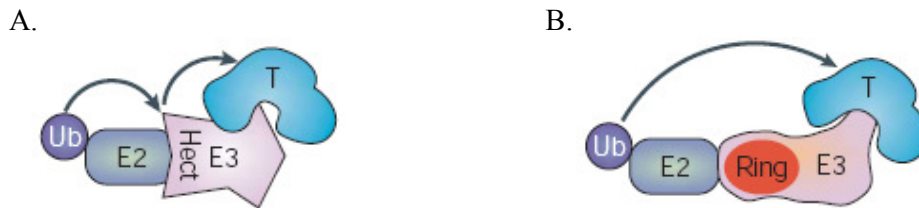


Figure 3: HECT-type and RING-type ubiquitin ligases. (A) HECT-type ubiquitin ligases load the ubiquitin onto itself after binding the E2 before transferring the ubiquitin to the target. (B) RING-type ubiquitin ligases bind E2 which transfer the ubiquitin directly to the target protein (Sullivan et al., 2003).

Homologous to E6-AP carboxyl terminus (HECT)-type E3s differ from the three other ubiquitin ligase families by possessing built-in catalytic activity and by executing the ubiquitylation reaction differently from the others. The family contains a large C-terminal HECT domain of approximately 350 amino acids with an E2 binding site and an active cysteine, separated by a short flexible linker (Kee and Huibregtse, 2007). To ubiquitylate substrates, the HECT-type E3 binds an E2 and load the ubiquitin onto its own catalytic cysteine to form a thiolester with ubiquitin before transferring it onto the substrate (Bernassola et al., 2008). See Figure 3A. The first characterized HECT-type E3 ligase is the E6-associated protein (E6-AP). Other HECT-type E3s are Huwe1, Nedd4, Itch and Smurfs.

The really interesting new gene (RING)-type E3s lack the catalytic site of HECT-type ligases and function primarily as scaffolds orienting the E2-ubiquitin complex and the target protein for ubiquitin transfer (Wang and Pickart, 2005). The RING-finger domain, which contains eight cysteine and histidine residues to coordinate two zinc molecules, mediates the interaction with the E2, and it is the E2 that transfer ubiquitin directly to the target protein. See Figure 3B. It has become clear that many, but probably not all, RING-finger-containing proteins function as ubiquitin ligases. RING-type E3s include both monomeric proteins like Mdm2 and multimeric protein complexes like SCF and APC.

U-box-type E3s are predicted to be very similar in three-dimensional structure as RING-type E3s despite the lack of a zinc binding domain. Instead, the E3s contain a U-box domain which consists of approximately 70 amino acids. Most, if not all, U-box proteins interact with molecular chaperones or co-chaperones which suggest a role mainly in quality control of accumulated abnormal proteins (Hatakeyama and Nakayama, 2003).

PHD-type E3s is a novel class of E3 ubiquitin ligases which contain a plant homeo-domain, a specialized form of zinc finger that is related to the RING-finger domain. It is not known how many of PHD-finger containing proteins are E3 ligases. These E3 ligases control the trafficking and/or degradation of target proteins involved in different cellular functions outside the nucleus. Examples of PHD-type E3s are MIR and MK3, which are membrane-bound (Coscoy and Ganem, 2003).

The RING-finger and PHD-finger share similarities in structure and size with classical zinc-fingers which apply four cysteine and histidine residues to coordinate a single zinc atom. Originally, zinc-fingers were only considered as DNA binding motifs, but are now known to also recognize RNA and proteins (Gamsjaeger et al., 2007).

Examples of ubiquitin ligases involved in DDR

Examples of ubiquitin ligases involved in DDR are Mdm2, BRCA1/BARD1, FANCL, Rad18 and RNF8. SCF and APC are multi-subunit ubiquitin ligases involved in the regulation of cell cycle progression and cell cycle checkpoints and are described later. All of these ubiquitin ligases are RING-type E3s except FANCL which is a PHD-type E3 (Meetei et al., 2003). Mdm2 has a C-terminal RING-finger with activity against p53 and itself, and is one of several ubiquitin ligases involved in regulating p53 activity ((Honda and Yasuda, 2000;Fang et al., 2000). BRCA1 contains a RING-finger and forms a heterodimer with another RING-type E3, BARD1. The BRCA1/BARD1 complex is found to be able to autoubiquitylate through K6 chain formation (Wu-Baer et al., 2003). BRCA1/BARD1 is involved in DNA repair, and a newly identified substrate is the chromatin bound protein CtIP (Yu et al., 2006) which is required for resection of DNA ends during homologous recombination (Sartori et al., 2007). RNF8 was recently discovered to be involved in DDR by facilitating local ubiquitylation of histones involved in recruitment of repair proteins to site of damage (Huen et al., 2007;Kolas et al., 2007;Mailand et al., 2007). This ubiquitin ligase is also described later. FANCL is involved in monoubiquitylation of FANCD2 during normal replication and in response to DNA damage (Meetei et al., 2004;Taniguchi et al., 2002). Upon treatment with DNA damaging agents, PCNA becomes ubiquitylated by E2 Rad6 and E3 Rad18 (Hoege et al., 2002;Kannouche et al., 2004).

Deubiquitylating enzymes

Deubiquitylating enzymes (DUBs) reverse the action of ubiquitin ligases by hydrolyzing the isopeptide bond between ubiquitin and the substrate protein or between two ubiquitin moieties. The enzymes are involved in keeping the proteasome free of ubiquitin, in the recycling ubiquitin and in proofreading ubiquitin-protein conjugates. Furthermore, they are involved in processing inactive ubiquitin precursors which are synthesized with a C-terminal extension or linear precursor fusion proteins (Amerik and Hochstrasser, 2004;D'Andrea and Pellman, 1998;Wilkinson, 1997). DUBs are believed to be functionally as important as ubiquitin ligases, but much less are known about them. The enzymes are less characterized in terms of function, specificity and regulation of their activity. Furthermore, only few substrates have been assigned. But it is known, that the DUBs are involved in many cellular functions and in diseases such as cancer and neurodegeneration (Nijman et al., 2005b).

DUBs belong to the protease superfamily, which has an estimated 553 members (Puente et al., 2003). The majority of DUBs are cysteine proteases and a few are metallo proteases. The DUBs that are cysteine proteases can be further divided into four groups based on their ubiquitin protease domain which are Ubiquitin specific protease (USP), ubiquitin C-terminal hydrolase (UCH), Ovarian Tumor-related protease (OTU) and Machado-Joseph disease protease (MJD). All DUBs that are metalloproteases have an ubiquitin protease domain called JAMM (Jab1/MPN/Mov34 metalloenzyme). In a search for genes whose transcript encodes one of these five ubiquitin protease domains, 95 putative DUBs were found. 79 of these are believed to be expressed and functional

(Amerik and Hochstrasser, 2004;Nijman et al., 2005b). The catalytic activity of cysteine proteases by definition depends on the thiol group of a cysteine in the catalytic domain which is deprotonated by an adjacent histidine. Together with aspartate, the three residues make up a catalytic triad. In catalysis, the cysteine makes a nucleophilic attack on the carbonyl of the isopeptide bond between the target protein and ubiquitin. This results in a release of the target protein and formation of a covalent bond between the DUB and ubiquitin. Upon contact with a water molecule, the ubiquitin is released from the DUB (Nijman et al., 2005b).

The USP group of DUBs is the largest, most diverse and best studied. The catalytic domain of this group of DUBs contain two short and well conserved motifs named the Cys and His boxes which are critical for catalysis. The two boxes are separated by 300-800 unrelated amino acids which may serve a regulatory function. Many interesting protein domains including ubiquitin binding, ubiquitin-like or zinc-fingers have been identified in the N-terminal and/or C-terminal part of the catalytic domain and are likely involved in the substrate specificity of the DUBs. USP DUBs appear to be specific for proteins modified with ubiquitin (Amerik and Hochstrasser, 2004;Nijman et al., 2005b).

The UCH group of DUBs is more conserved in amino acid sequence than the USP DUB group and has four members. The UCH DUBs were the first described but their specific functions are still poorly understood. They are believed to be involved in the recycling of ubiquitin when ubiquitin is inappropriately conjugated to intracellular nucleophiles like glutathione or polyamines. Furthermore, they are believed to be involved in the processing of ubiquitin from polyubiquitin precursors or when fused to ribosomal protein precursors (Amerik and Hochstrasser, 2004;Nijman et al., 2005b).

The OTU group of DUBs was discovered in a bioinformatic study (Makarova et al., 2000). The catalytic triad in a recently solved OTU structure was incomplete and is stabilized by another method using a hydrogen bonding network instead (Nanao et al., 2004;Balakirev et al., 2003).

The MJD group of DUBs includes Ataxin-3 and a number of Ataxin-3-like proteins. The enzymes have low sequence similarity with other DUBs but NMR structures show conservation in the overall arrangement of the catalytic triad. Ataxin-3 has shown deubiquitylating activity which has not yet been demonstrated for the other MJD DUBs, and their biological functions remains unknown (Amerik and Hochstrasser, 2004;Nijman et al., 2005b) .

Metallo proteases have no active cysteine residue but instead two histidines and an aspartate. Together with a Zn^{2+} bound polarized water molecule, the residues generate a non-covalent intermediate with the substrate. The ubiquitin is also released from DUB upon contact with a water molecule (Ambroggio et al., 2004;Nijman et al., 2005b). The JAMM group of DUBs includes Rpn11/POH1 which is a subunit of the 19S proteasomal lid with activity against ubiquitylated proteins (Verma et al., 2002). Another DUB in this group is AMSH which associates with a protein involved in regulating receptor sorting in the endosome (McCullough et al., 2004).

Examples of deubiquitylating enzymes in DDR

Examples of DUBs involved in DDR are USP1, USP3, USP7 and USP28. USP1 is believed to be the DUB of ubiquitylated FANCD2 and PCNA (Huang and D'Andrea, 2006;Nijman et al., 2005a). USP3 has been suggested to deubiquitylate histone H2A and H2B and is required for S-phase progression and genome stability (Nicassio et al., 2007). USP7 is suggested to be the DUB of both p53 and its ubiquitin ligase, Mdm2, and is involved in regulating p53 stability (Li et al., 2002;Li et al., 2004;Cummins and Vogelstein, 2004). USP28 was found to be a DUB of Claspin and counteracts its APC^{Cdh1} mediated degradation in response to DNA damage in G2 (Bassermann et al., 2008).

Ubiquitin-binding domains

Ubiquitin-binding domains (UBDs) are a group of protein domains that non-covalently bind to ubiquitin. The domains are small with 20-150 amino acids and are structurally diverse with different biological functions. Ubiquitin-binding proteins apply UBDs to directly interact with monoubiquitin or polyubiquitin chains. The domains can be found in enzymes involved in ubiquitylation and deubiquitylation. Furthermore, they can be found in proteins that recognize and interpret signals from ubiquitylated substrates. The presence of a UBD in a protein indicates that it can interact with ubiquitin or ubiquitylated proteins and it might be regulated by ubiquitylation itself (Hicke et al., 2005). In 2006, 16 different UBDs had been identified and the domains bind various surfaces on ubiquitin (Hurley et al., 2006). A newly identified protein with UBDs involved in DDR is Rap80 which is involved in targeting BRCA1 to site of DNA damage. The protein has two UIM domains which bind polyubiquitin chains (Wang et al., 2007;Sobhian et al., 2007;Kim et al., 2007a).

3.2 The Cell Cycle

The cell cycle is the process where a cell duplicates its entire DNA and divides into two new genetically identical daughter cells. The cell cycle of mammalian cells consists of four phases which are synthesis (S), mitosis (M) and two gap phases (G1 and G2). See Figure 4A. In S-phase, which is the longest phase of the cell cycle, the entire DNA is replicated to produce two identical copies. After DNA replication, the DNA is segregated and divided into two new cells in the mitosis phase. An overview of the different phases of mitosis is given in Figure 4B. The S and M phases are separated with two gap phases G1 and G2, which gives the cell time to grow and monitor internal and external signals and to ensure proper timing for entering S or M phase. The interphase of the cell cycle covers G1, S and G2, and a round of cell division generally takes about 24 hours in rapidly cycling cells in culture.

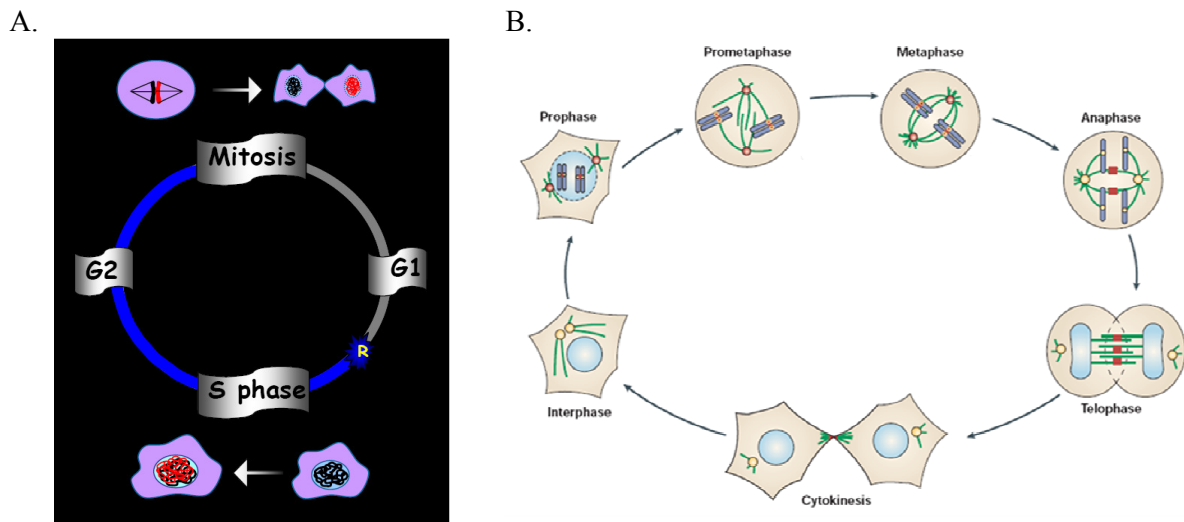


Figure 4: An overview of the cell cycle. (A) The different phases of the cell cycle include the S phase where the cell replicates its entire genome, mitosis where the cell divide into two identical daughter cells and the two gap phases G1 and G2. R represents the restriction point from where the cell commit to a round of replication and division. (B) Detailed overview of the different phases of mitosis where the cell carefully divides the replicated DNA into two new daughter cells. Modified from (Barr et al., 2004).

The cell cycle is a tightly regulated process and in multicellular organisms, most eukaryotic cells only divide during development and when dead cells need renewal in developed organism. In a developed organism, most cells are kept in a non-replicative stage (quiescent stage, G0) and only divides when the cellular and tissue environment are favorable and only in the presence of mitogenic factors, which signals the need for cell proliferation. Cells harbor several mechanisms to sense the presence of mitogenic factors as well as anti-proliferative signals or damage that prevents cell cycle progression. Extracellular signals are sensed through transmembrane cell surface receptors which are coupled to intracellular signaling circuits. At the onset of the cell cycle in G1, mitogenic factors stimulate the expression of proto-oncogenes such as Ras and Myc, which results in transcriptional regulation leading to increased proliferation (Berthet and Kaldis, 2007; Bartek and Lukas, 2001).

A special feature of cell cycle is the unidirectionality. As soon as the cells has committed to duplication, the cell goes through the entire cycle and through the different cell phases without returning to a previous one. This is ensured by the degradation of proteins that are needed in previous stages. Many proteins undergo proteolysis at different cell cycle transitions and slowly synthesized proteins are rapidly degraded which provides an irreversible mechanism that blocks the way back,

thus driving the cell cycle forward. The gradual accumulation and abrupt degradation of cyclins cause oscillations in cdk activities which are the major regulator of the cell cycle progression (Vodermaier, 2004). Cell cycle checkpoints are networks of signaling pathways that monitor the order and quality of the cell cycle. With checkpoints, cells actively halt progression through the cell cycle until it can ensure that an earlier process such as DNA replication or mitosis is complete. Besides the DNA damage checkpoints that recognize and respond to DNA damage at the G1/S, intra S and G2/M, the cells sense the fidelity of DNA replication (replication checkpoints) and make sure all chromosomes are aligned before entering mitosis (spindle checkpoint in metaphase). The cell cycle checkpoints are generally not essential for cell cycle progression but are best viewed as accessory braking systems to avoid accumulation of mutations or large scale changes in chromosome composition, which can promote cancer (Hartwell and Weinert, 1989; Nyberg et al., 2002).

Cyclins and cdks

Cyclin-dependent kinases (cdks) are a family of serine/threonine kinases which are dependent on association with cyclins to become active. Cyclins were discovered as proteins that accumulate progressively through interphase and disappear abruptly at mitosis during each cell cycle (Hunt, 2004; Evans, 2004). They were later found to function in mitosis as well as other phases of the cell cycle. Different cyclins are present in different phases of the cell cycle and are regulated by protein synthesis and degradation. The level of cdks remains constant but the activity oscillates throughout the cell cycle due to changes in the presence of cyclins. Phosphorylation of specific targets by cyclin-cdk complexes is crucial for progression through the cell cycle in a timely manner. Cdks regulate important processes like DNA replication, mitosis, centrosome/spindle pole duplication or chromosome condensation either directly or through various effector kinases (Morgan, 1997; Fung and Poon, 2005).

In *S. cerevisiae*, only one cdk, Cdc28, associate with several cyclins to regulate the cell cycle. Mammalian cells possess multiple cdks which are each dependent on a particular cyclin. The first family of cyclins to be induced is the D-type to activate Cdk4/Cdk6. The complexes phosphorylate and activate the retinoblastoma protein (Rb) which promotes progression through the restriction point (R), which is at late G1 and serves as a mark for the decision to commit to replication of the genome and division. From here, cells switch from mitogen-dependent growth to largely growth factor independent progression into S and beyond. Phosphorylation of Rb activates E2F dependent transcription of genes including Cyclin E and other factors required for DNA replication. Cyclin E activates Cdk2 to initiate S-phase which is maintained by Cyclin A-Cdk2. Mitosis is driven by the activity of Cyclin B-Cdk1 and to a lesser extent Cyclin A-Cdk1 (Bartek and Lukas, 2001; Berthet and Kaldis, 2007; Morgan, 1997). An overview of the different cyclin-cdk complexes activated during the different stages of the cell cycle is given in Figure 5.

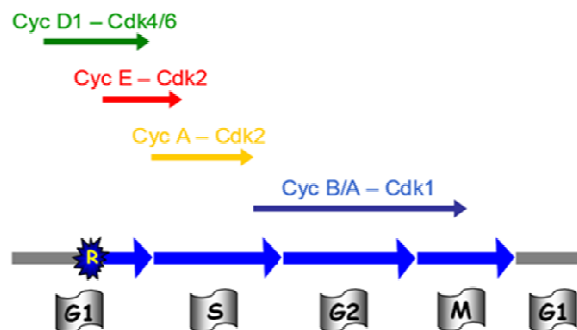


Figure 5: Activation of cyclin-cdk complexes during the cell cycle. Cyclin D1-Cdk4/6 activity mediates passage through the restriction point (R). Cyclin E-Cdk2 initiates S-phase which is maintained by Cyclin A-Cdk2. Cyclin B-Cdk1 and Cyclin A-Cdk1 drives the mitosis.

Cdk inhibitors (CKIs) are negative regulators of cyclin-cdk complexes and can be divided into two families depending on their structure and target which are the INK4 proteins and the CIP/KIP proteins. INK4 proteins specifically bind Cdk4 and Cdk6 to inhibit their catalytic activity. CIP/KIP proteins include p21, p27, and p57 and inhibit the activities of Cyclin D-, E- and A-cdk complexes by binding both cyclin and cdk subunits (Morgan, 1997; Sherr and Roberts, 1999; Wade Harper and Elledge, 1996).

Ubiquitin dependent proteolysis as a key determinant of cell cycle transitions

The amount of cyclins, CKIs and many other cell cycle regulators oscillate during the cell cycle as a result of accumulation and degradation. The degradation of these proteins is mediated by the ubiquitin-proteasome system. Two major ubiquitin ligases are responsible for the periodic proteolysis of many regulators of the cell cycle. These are the Anaphase Promoting Complex (APC) and the Skp1-Cul1-F-box protein (SCF) complex. The SCF and APC complexes are structurally similar, each consisting of invariable core subunits and a variable substrate recognition subunit. The different subunits are presented in Figure 6 A and B. Despite structurally similar, the APC and SCF have different cellular functions.

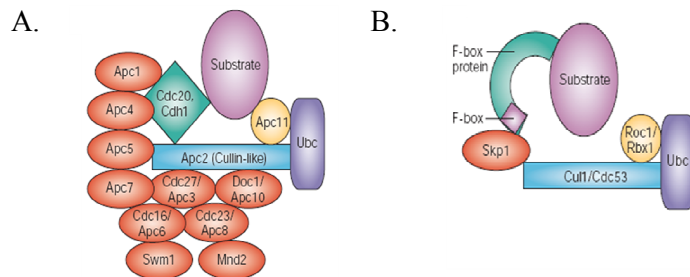


Figure 6: The APC and SCF ubiquitin ligase complexes together with an E2 (Ubc). (A) The APC complex consist of the RING-finger protein Apc11, the scaffold protein Apc2, many other subunits which are shown in red and the variable adaptor protein which are Cdc20 or Cdh1. (B) The SCF complex consist of the RING-finger protein Roc1/Rbx1, the scaffold protein Cul1/Cdc53, the adaptor protein Skp1 and a variable F-box protein (Reed, 2003).

SCF complex

The SCF complex consist of Rbx1 (RING-finger protein), Cul1 (scaffold protein) and Skp1 (adapter protein) and a variable F-box protein which bind to Cul1. Approximately 70 F-box proteins have been identified in humans and at least three are involved in cell cycle control. These are Skp2, Fbw7 and β TrCP, which gives substrate specificity to SCF. The SCF was originally thought to function mainly at the G1-S transition but is active from late G1 to early M. The activity of SCF is constant and SCF only ubiquitylates specific phosphorylated target proteins in most cases. These include G1/S cyclins and certain CKIs controlling S-phase initiation (Nakayama and Nakayama, 2005; Nakayama and Nakayama, 2006). As illustrated in Figure 7, the three F-box proteins are involved in both cell cycle promotion and cell cycle inhibition.

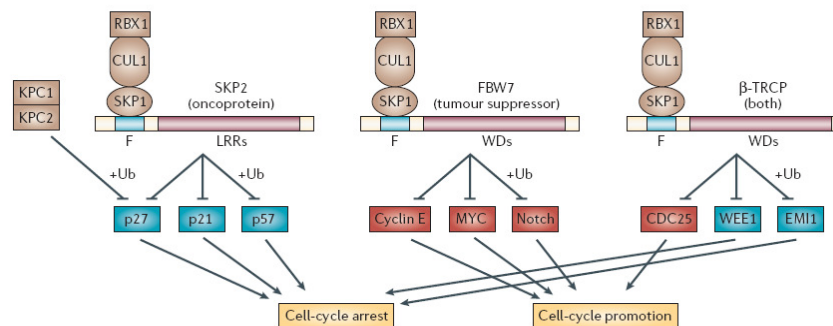


Figure 7: Functions of Skp2, Fbw7 and β TrCP. The three different SCF F-box proteins Skp2, Fbw7 and β TrCP and their major targets are shown. Red boxes indicate cell cycle promoters and blue boxes cell cycle inhibitors. Skp2 promotes cell cycle through targeting CKIs. Fbw7 inhibits the cell cycle through targeting cell cycle promoters. β TrCP both promotes and inhibits the cell cycle. F: F-box protein, LRR: Leucine-rich repeats, Ub: Ubiquitin, WDs: WD40 repeats (Nakayama and Nakayama, 2006).

Skp2

The F-box protein Skp2 is involved in executing entry into S-phase by targeting the cdk inhibitors p21 and p27 for degradation (Carrano et al., 1999; Bornstein et al., 2003). Many other proteins are reported as targets including p57 which is a tumor suppressor involved in keeping cdks inactive in G0 and is degraded upon transition into G1 (Kamura et al., 2003). APC^{Cdh1} preserves the inhibition of Cdk1 and Cdk2 in G1 by targeting Skp2 for degradation. When cells commit to enter the cell cycle, accumulated Emi1 inhibit APC^{Cdh1} during S and G2, which then releases Skp2 to execute its functions (Cardozo and Pagano, 2004).

Fbw7

The F-box protein Fbw7 has shown to be a tumor suppressor by targeting several growth promoters including Cyclin E, Myc, Jun, Notch1 and Notch4 for degradation. Cyclin E is involved in the G1/S transition and is targeted for degradation in a phosphorylation dependent manner as is the case for the other Fbw7 substrates (Nakayama and Nakayama, 2005; Welcker and Clurman, 2008).

βTrCP

βTrCP proteins are highly evolutionally conserved and involved in many signaling pathways. The consensus sequence recognized by βTrCP is the DSGX_{2+n}S destruction motif where the serines are phosphorylated by specific kinases (Hattori et al., 1999). βTrCP targets β-catenin and IκBα for ubiquitylation and degradation. β-catenin is an important mediator of Wnt signaling and IκBα is an inhibitor of the transcription factor NFκB. Other substrates include IκBβ, IκBε, transcription factor Atf4, p105, p100, subunits of NFκB and the disc large tumor suppressor.

βTrCP also exerts direct control of cell cycle regulators including the two negative regulators Emi1 and Wee1 and the positive regulator Cdc25. Emi1 accumulates in S phase and G2 where it blocks the degradation of Cyclin A and Cyclin B by inhibiting the activity of both APC^{Cdc20} and APC^{Cdh1} (Reimann et al., 2001a; Reimann et al., 2001b). Degradation of Emi1 is therefore necessary for activation of APC in late mitosis. In early mitosis, Emi1 is phosphorylated by Plk1 on a DSGXXS motif and is subsequently recognized by SCF^{βTrCP} which leads to its degradation and contributes to activation of APC (Moshe et al., 2004). The tyrosine kinase Wee1 phosphorylates and inhibits the activity of Cyclin B-Cdk1 in S and G2, hereby functioning as a negative regulator of cell cycle progression. To allow rapid activation of Cdk1, Wee1 needs to be down-regulated at the onset of mitosis. Wee1 is phosphorylated by Plk1 at two phosphorylation sites without having a canonical βTrCP destruction motif. Wee1 are then recognized by SCF^{βTrCP} and degraded (Watanabe et al., 2004). βTrCP also regulates the abundance of the phosphatase Cdc25A. E2F and c-myc transcription factors stimulate transcription of Cdc25A in mid to late G1. Through activation of Cdk1 and Cdk2 by dephosphorylation, Cdc25A induce S-phase entry, progression through S and mitosis. Cdc25A is phosphorylated by Chk1 upon DNA damage in S phase and G2 followed by phosphorylation of its DSGXXXXS destruction motif by an unknown kinase, allowing recognition and ubiquitylation by βTrCP. Both the constitutive and DNA damage induced turnover of Cdc25A depends on SCF^{βTrCP}. (Busino et al., 2003; Jin et al., 2003).

More recently, the adaptor protein Claspin was also identified as a substrate of βTrCP. Claspin is required for stabilization and ATR-mediated activation of Chk1. At the onset of mitosis or during recovery from genotoxic stress, Claspin becomes phosphorylated on its N-terminal βTrCP recognition motif, DSGXXS, by Plk1 leading to its ubiquitylation by SCF^{βTrCP}, and degradation by the proteasome (Mailand et al., 2006; Peschiaroli et al., 2006; Mamely et al., 2006). The function and regulation of Claspin will be described in more details later.

APC complex

Cdk activity drives cell cycle progression as far as metaphase, but to enter anaphase and beyond, the activity of APC is needed (Sullivan and Morgan, 2007). The APC consist of Apc11 (Rbx1-related RING-finger protein), Apc2 (Cul1-related scaffold protein) and at least 11 other components without defined role together with a variable activator. Two different activators functioning in the cell cycle are Cdc20 and Cdh1. Additional APC activators are found to function in non-dividing cells and during meiosis (Nakayama and Nakayama, 2006). The activity of APC changes through the cell cycle and its activity are turned on mainly by binding of the activating subunits to the complex. APC is active from early mitosis until end of the G1 phase. Interaction of APC with Cdc20 requires cdk phosphorylation on multiple sites of APC while interaction with Cdh1 is irrespective of APC phosphorylation. Instead cdk phosphorylation of Cdh1 is required for association with APC (Vodermaier, 2004). APC recognises specific motifs in the primary sequence of target proteins which includes mitotic cyclins and other regulators of mitosis. The best studied APC degradation motifs are the destruction box (D box) with the consensus sequence RXXLXXXXN, which is recognized by both APC^{Cdc20} and APC^{Cdh1}, and the KEN box with the consensus sequence KENXXXXN, which is also recognized by APC^{Cdh1} (Wäsch et al., 2005).

Cdc20

Cdc20 is expressed in G2 before entering M phase but the activity of APC^{Cdc20} is suppressed until all chromosomes are attached correctly to mitotic spindles and aligned, also known as the spindle checkpoint. Thus Cdc20 becomes active from early mitosis and is active until the M to G1 transition, where it is targeted for degradation by APC^{Cdh1}. The major targets of Cdc20 are securin and mitotic cyclins. Degradation of securin results in initiation of chromosome segregation by unleashing the protease Separase which destroys sister-chromatid cohesions. Degradation of mitotic cyclins (Cyclin A and B) results in inactivation of cdks, allowing phosphatases to dephosphorylate many cdk substrates, which are required for the events of anaphase and telophase. These events include chromosome and spindle movements, spindle disassembly, reformation of nuclei and decondensation of chromatin (Sullivan and Morgan, 2007).

Cdh1

The activity of Cdh1 during cell cycle is regulated by phosphorylation and dephosphorylation. Cdh1 is phosphorylated by cdks during S, G2 and early M, preventing access of Cdh1 to the core APC subunits. In late M and G1 phases, Cdh1 is activated due to a drop in cdk activity and dephosphorylation by Cdc14 which allows Cdh1 to interact with APC core subunits and target proteins for degradation. Cdh1 has a broader specificity than Cdc20 and the many protein targets include mitotic cyclins (Cyclin A and B), mitotic kinases like Plk1 and Aurora kinases, proteins involved in spindle function like kinesins and proteins involved in DNA replication like Cdc6 and geminin. Cdh1 hereby acts mainly to ensure ordered mitotic exit, cytokinesis and to prevent premature S phase activation.

Interplay between SCF and APC

There is indication of a tight interplay between the SCF and APC and that the two complexes control each other. SCF^{βTrCP} mediates the degradation of Emi1 in prometaphase which is an inhibitor of APC and contributes to its activation (mentioned above). During G1, APC^{Cdh1} promotes proteolysis of Skp2 and its cofactor Cks1 thus preventing premature degradation of SCF^{βTrCP} substrates. Furthermore there is a high level of cross talk between the two complexes. An example is Cdc25A which contains both a KEN box and a phosphodegron, and is degraded by the APC^{Cdh1} at the end of mitosis, and by SCF^{βTrCP} in S and G2, as mentioned above (Busino et al., 2003).

Cell cycle as a three layered process

An overview of the different layers of the cell cycle are presented in Figure 8. Overall, the cell cycle is driven by and regulated by the execution of phosphorylation and ubiquitylation reactions. The events of the cell cycle including replication and mitosis which are qualitatively controlled by phosphorylation which are largely dependent on cdks, cyclins (accelerators) and CKIs (brakes). These accelerators and brakes are quantitatively regulated by ubiquitylation involving ubiquitylating enzymes (Nakayama and Nakayama, 2006).

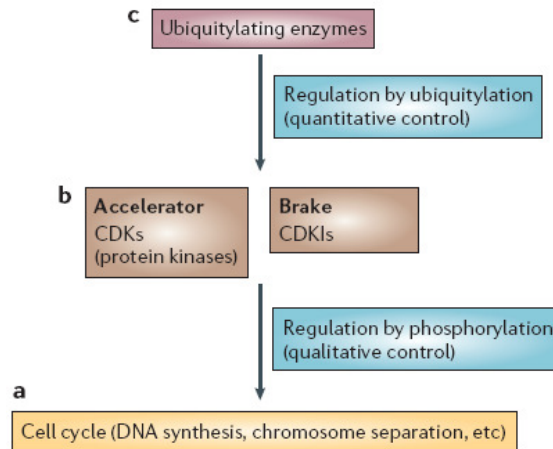


Figure 8: Cell cycle viewed as a three layered process. The cell cycle can be viewed as a three layered process in which the cell cycle progression (A) with DNA synthesis and mitosis is regulated qualitatively by phosphorylation. The phosphorylation activities is executed by the cdks (B) influenced by CKIs which are furthermore regulated quantitatively by ubiquitylating enzymes (C) (Nakayama and Nakayama, 2006)

3.3 Cellular responses to DNA damage

Preserving the integrity of the genome is demanding due to the continuous threat of adverse genetic changes from a plethora of DNA lesions. DNA lesions can be caused by genotoxic insults from the environment but also from the cells own metabolism. If DNA lesions are not repaired, they can result in gene mutations which eventually can lead to development of cancer. Indeed, the cellular responses to DNA damage constitute one of the most important fields in cancer biology (Bartek et al., 2004).

Cells have evolved to deal with the different sources of DNA damaging agents through the development of elegant mechanisms that detect and repair damage to the genome. The DNA damage response (DDR) orchestrates the appropriate repair and resolution of replication problems and coordinates these processes with the ongoing cellular physiology (Harper and Elledge, 2007). Upon identification of damaged or abnormally structured DNA, eukaryotic cells directly halt the cell cycle progression, repair the DNA or undergo programmed cell death if the damage is too excessive. This coordination is critical for cell survival, especially when DNA replication is perturbed (Matsuoka et al., 2007). The cellular response to DNA damage depends on the type of damage and which cell cycle phase the cells are in, and can be considered more as flexible networks than strict linear pathways.

Types and sources of DNA damage

DNA damaging agents directly damage bases or break the phosphodiester backbone on which the bases reside. Types of DNA damage are mainly base loss, base modifications, single strand breaks (SSB), double strand breaks (DSB), inter and intra strand crosslinks. At the cellular level, DNA damages can hamper transcription of genes, replication of DNA and result in cell cycle arrest, chronic mutations and cell death. At the organism level, DNA damages have been implicated in carcinogenesis, genetic disorders and in ageing (Altieri et al., 2008). DNA lesions can be caused by environmental insults such as ionizing or ultra violet radiation, various genotoxic compounds and drugs. They can also be caused by endogenous genotoxic insults from reactive cellular metabolites naturally occurring in the cell. The chemical events that lead to DNA damage include attack by water, reactive oxygen species and other reactive metabolites (Hoeijmakers, 2001; De Bont and van Larebeke, 2004; Lindahl and Wood, 1999).

The bonds between bases and sugar residues in the backbone are relatively labile and attack by water causes hydrolysis of the glycosylic bond and results in formation of mutagenic abasic sites (De Bont and van Larebeke, 2004). Reactive oxygen species are one of the major sources of DNA damage and are formed continuously in living cells. ROS comes from mitochondria, peroxisomes, cytochrome P450 enzymes, antimicrobial oxidative burst of phagocytic cells and other biological reactions. The species include superoxide, hydrogen peroxide, hydroxyl radical and singlet oxygen which attacks the bases or deoxyribose moieties for oxidation and result in adducts, cross links, single and double strand breaks. The most abundant damage from ROS is formation of the oxidized base 8-oxo-Gua (8-oxo-7,8-dihydroguanine) which results in mispairing of guanine to adenine. Deleterious ROS are also produced upon exposure of cells to various chemical and physical agents such as certain drugs, UV light and IR. Exposure to reactive nitrogen species like peroxynitrate results in deamination of DNA bases and other base modifications are formed by reaction with alkylating and methylating agents. Furthermore, platinum compounds like cisplatin is able to covalently bind to DNA bases, producing intra and inter strand cross links (Altieri et al., 2008; De Bont and van Larebeke, 2004; Lindahl and Wood, 1999).

Exogenous sources of DNA damage include ultraviolet (UV) and ionizing radiation (IR). Ionizing radiation of cells can produce highly reactive radical species and cause a broad spectrum of lesions similar to those produced by ROS and include damage to purine and pyrimidine rings, AP sites and strand breaks. Radiation by the UV component of sunlight primarily generates the helix-distorting lesions cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts (6-4PPs) from direct excitation of nucleobases. CPDs result in mainly T pairing with T and 6-4PPs result mainly in T pairing with C (Altieri et al., 2008; Lindahl and Wood, 1999).

To protect the genome, cells need to detect all types of DNA damage and structural alterations including nicks, gaps, strand breaks and the many alterations that block DNA replication. The possibly deadliest type of DNA damage is DSBs, which can result in mutations, translocations, chromosome loss and cell death, if not repaired correctly (Hoeijmakers, 2001; Pastink et al., 2001). An overview of the DNA damaging agents, the DNA lesions they generate and the DNA repair mechanisms applied for removal of the damage is given in Figure 9.

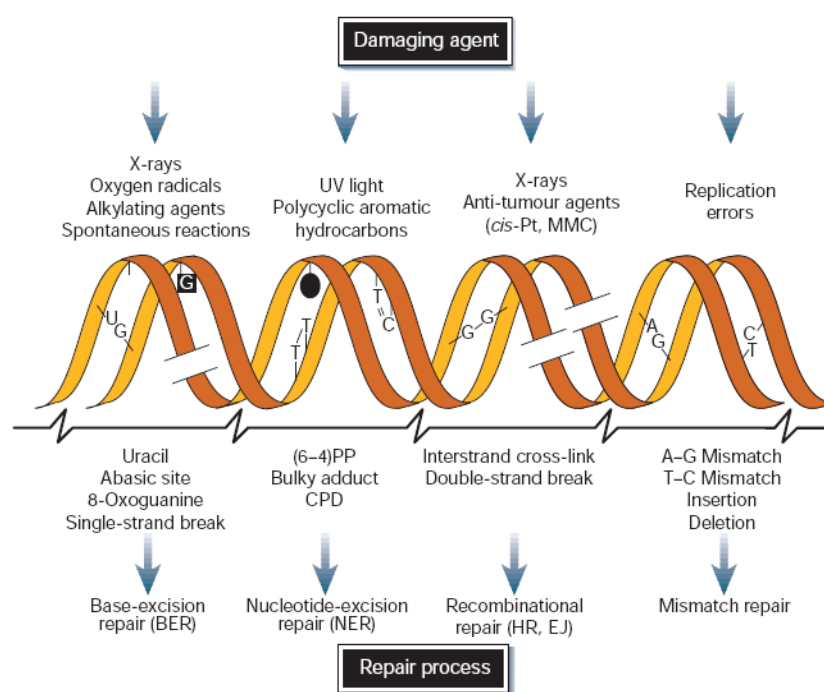


Figure 9: DNA damaging agents and repair mechanisms. (Top) The most common DNA damaging agents including the DNA-cross-linking agents cisplatin (Cis-Pt) and mitomycin C. (Middle) Examples of DNA lesions generated by the DNA damaging agents including (6-4) photoproducts ((6-4)PP) and cyclobutane pyrimidine dimers (CPD). (Bottom) The most relevant DNA repair pathways responsible for removal of the lesions including homologous recombination (HR) and end joining (EJ) (Hoeijmakers, 2001).

Repair pathways

To prevent the consequences of DNA damage on cell fate and to prevent loss of information, the cells have evolved a complex network of DNA repair pathways. More than 120 genes in the human genome have been found to be involved in repair mechanisms (Wood et al., 2005). The selection of repair pathway depends on the type of damage and which cell cycle phase the cell is in. The DNA double helix has the advantage of containing information in both strands. If one strand is damaged, the other strand is applied as template in the repair process. This makes repair of double strand breaks more demanding. The different repair pathways include base excision repair (BER) which repairs most small base modifications and nearly all oxidatively induced DNA lesions (except DSBs and

purine cyclonucleosides) and also SSBs (Altieri et al., 2008;Zharkov, 2008). Nucleotide excision repair (NER) removes photoproducts, bulky and helix distorting adducts, cross links and oxidative damage (Altieri et al., 2008;de Laat et al., 1999). DNA mismatch repair (MMR) maintains genetic integrity by correcting base substitution mismatches and insertion/deletion loop mismatches generated as a result of replication errors or as a result from a variety of internal and external stresses that escape the proofreading function of DNA polymerases (Altieri et al., 2008;Hsieh and Yamane, 2008). Furthermore two repair pathways, homologous recombination (HR) and non-homologous end joining (NHEJ), repairs double strand breaks (Hoeijmakers, 2001). HR repair of broken ends is generally accurate because the undamaged sister chromatid is used as a repair template. This also restricts HR to late S and G2. NHEJ joins DNA ends without the requirement for sequence homology, and NHEJ can function throughout the cell cycle, but plays an important role during G1, when no sister chromatid is available (Hendrickson, 1997;Khanna and Jackson, 2001).

In response to DSBs, the two phosphatidyl-inositol-3-OH kinase –like kinases, ATM and ATR are activated and work together. The cellular response to DSBs is described in details below to illustrate the initial phases of the DNA damage response.

The cellular response to DNA double strand breaks

Cells react to DSBs by rapidly recruiting a host of proteins to the damaged chromatin regions where some are involved in DNA repair while others trigger signaling pathways. DSBs are believed to be recognized by the multifunctional Mre11-Rad50-Nbs1 (MRN) complex (Petrini and Stracker, 2003). The initial interaction of MRN with DSBs is transient and recruits the ATM kinase to the damaged DNA by interaction between the HEAT repeats of ATM and the C-terminus of Nbs1 (You et al., 2005;Lukas et al., 2003;Falck et al., 2005). It is still at debate if it is the recruitment that activates ATM or ATM is already activated upon recruitment (You et al., 2005;Bakkenist and Kastan, 2003). In both cases, ATM is autophosphorylated very rapidly upon irradiation which causes dimer dissociation and initiates cellular ATM activity (Bakkenist and Kastan, 2003). The ATM-MRN complex triggers two main pathways to prepare the DNA for repair and to initiate checkpoint signaling (Bartek and Lukas, 2007) which are here described as local chromatin rearrangements and DSB resection. An overview of the two pathways is given in Figure 10.

Local chromatin rearrangements

In the first pathway, activated ATM phosphorylates the histone H2A variant H2AX on the conserved serine residue ser139 at its C-terminus. This allow the mediator protein Mdc1 to bind the phosphorylated H2AX (γ -H2AX) through its tandem BRCT domains (Stucki et al., 2005). Mdc1 is one of the first proteins to accumulate at DSB sites and is believed to be a “master regulator” by promoting retention of many other proteins at the site of damage (Stucki and Jackson, 2006) and shielding the C-terminal tail of γ -H2AX from premature dephosphorylation by phosphatases (Stucki et al., 2005;Stewart et al., 2003). Interaction between Mdc1 and γ -H2AX is required for efficient accumulation of MRN and phosphorylated ATM at sites of damage (Stucki et al., 2005;Goldberg et al., 2003). This facilitates other ATM dependent phosphorylation of substrates including Chk2 (Matsuoka et al., 2000), p53 (Canman et al., 1998) and Kap1 (Ziv et al., 2006). The retention of two adaptor proteins 53BP1 and BRCA1 are dependent on Mdc1 without direct interaction. Mdc1 have been proposed to promote changes in the chromatin to allow binding of BRCA1 and allow accessibility of constitutively methylated residues on histones to bind 53BP1 (Bekker-Jensen et al., 2005;Huyen et al., 2004).

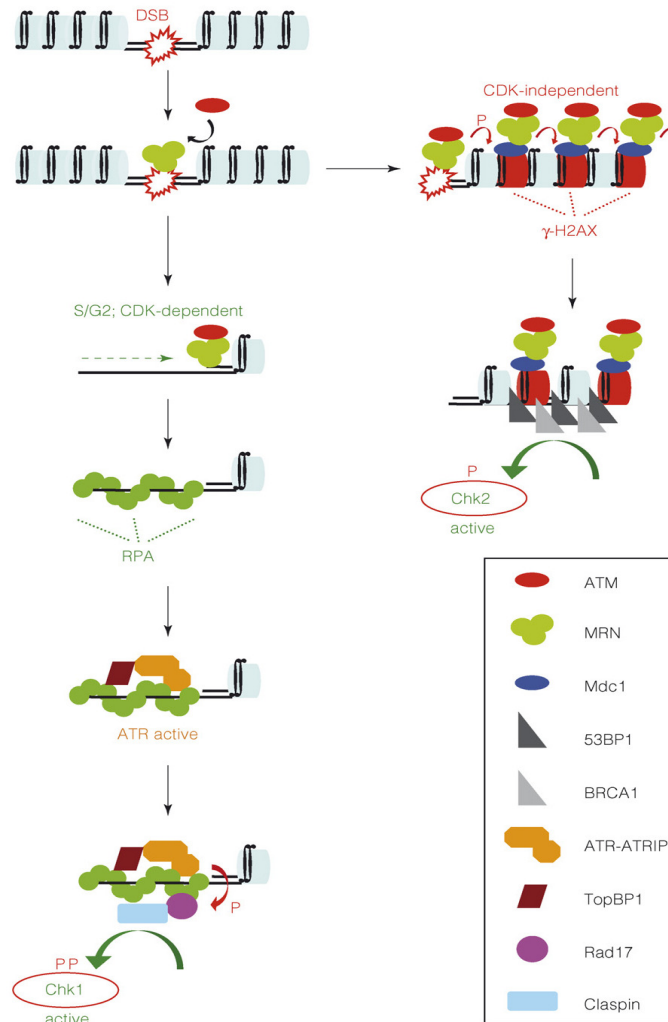


Figure 10: DNA damage response from DSBs. DSBs are recognized by the MRN complex which recruits ATM. Activated ATM initiates two pathways to rearrange the chromatin to facilitate repair and checkpoint signaling. In the first pathway shown to the right of the figure, ATM phosphorylates histone H2AX which allows recruitment of Mdc1, MRN, ATM and activation of downstream targets including Chk2. The chromatin rearrangement facilitates recruitment of BRCA1 and 53BP1. In the other branch of the pathways, MRN and ATM facilitate resection of the DSB to allow recruitment of RPA followed by binding of ATR/ATRIP, TopBP1, Rad17 and Claspin. ATR is then able to activate downstream targets including Chk1 which requires Claspin (Bartek and Lukas, 2007).

BRCA1

BRCA1 contains an N-terminal RING-finger domain and tandem BRCT (BRCA1 C-terminal) domains at its C-terminus. BRCA1 dimerizes through its RING-finger domain with BARD1 (BRCA1-associated RING domain protein) to form an E3 ubiquitin ligase (Mallery et al., 2002). The BRCT domains are phosphoserine- or phosphothreonine-binding motifs which are also found in Mdc1 and 53BP1. BRCA1 is a tumor suppressor and many disease-causing mutations are detected within the RING and BRCT domains. Mutations in BRCA1 are associated with an increased risk of breast and ovarian cancer (Bochar et al., 2000).

Mutations in the BRCT domains of BRCA1 disrupt its recruitment to the site of DSBs (Greenberg et al., 2006). The BRCT domains bind three different proteins in a mutually exclusive manner which are BACH1 (Cantor et al., 2001), CtIP (Yu et al., 1998) and the newly identified Abraxas (Wang et al., 2007). Abraxas was also found to bind Rap80 which contains a tandem ubiquitin-interacting

motif domain, UIM (Wang et al., 2007). Rap80 co-localize with γ -H2AX and BRCA1 at DNA breaks in cells exposed to IR. Abrogation of IR-induced co-localization of Rap80 UIM deletion mutants suggested that Rap80 gains access to double strand breaks by cooperative UIM binding to ubiquitin or polyubiquitin (Kim et al., 2007a; Sobhian et al., 2007; Wang et al., 2007). GST-bound Rap80 was found to preferentially bind K63- and possibly K6 but not K48 linked ubiquitin polymers (Sobhian et al., 2007). Knockdown of Mdc1 or Rap80 each reduced the intensity of BRCA1 immunostaining at stripes and Rap80 recruitment to laser stripes was reduced in cells depleted of Mdc1. Together the data suggest that Rap80 recruit BRCA1 through Abraxas to ubiquitin structures at DSBs in an Mdc1 dependent manner. Cells depleted of Abraxas or Rap80 exhibited hypersensitivity to IR, G2/M checkpoint defects and defects in HR repair. The effects were less severe than in BRCA1 depleted cells which suggest that Abraxas and Rap80 mediate a subset of BRCA1 functions. Another protein found to require Rap80 for recruitment to IRIF is the DUB BRCC36 which is found to have DUB activity toward K63 linked ubiquitin substrates (Sobhian et al., 2007).

53BP1

53BP1 contains tandem BRCT domains at the C-terminus, tandem Tudor domains and several (S/T)Q sites in the N-terminus (Charier et al., 2004; Rappold et al., 2001). 53BP1 was first identified in a yeast two hybrid screen as a protein that interacts with p53 through its tandem BRCT domains (Iwabuchi et al., 1994). 53BP1 has key role in both DNA damage response signaling, repair of DSBs and is required for both IR-induced intra-S-phase and G2/M phase checkpoints (Wang et al., 2002). Mice deficient of 53BP1 show increased radiation sensitivity and elevated tumor risk (Morales et al., 2003) and 53BP1 has earlier been suggested to be involved in NHEJ due to its involvement in class switch recombination of immunoglobulin heavy chains (Manis et al., 2004; Ward et al., 2004). Recently, 53BP1 was found to accumulate at deprotected telomeres and suggested to have a role in facilitating NHEJ repair reactions by increasing chromatin mobility that involve distant sites, including joining of dysfunctional telomeres (Dimitrova et al., 2008). Furthermore, 53BP1 is suggested to facilitate long range DNA end-joining during variable, diversity and joining (V(D)J) recombination (Difilippantonio et al., 2008).

53BP1 rapidly accumulates at sites of DSBs and is hyperphosphorylated in an ATM dependent manner (Rappold et al., 2001; Schultz et al., 2000). Recruitment of 53BP1 at site of damage is dependent on γ -H2AX and Mdc1 but independent of Rap80 (Bekker-Jensen et al., 2005; Kim et al., 2007a). The Tudor domains of 53BP1 have been suggested to bind methylated histone H3 or H4 to target 53BP1 to DSBs. Initially, 53BP1 was suggested to bind H3 lysine K79 (H3-K79) from biochemistry and cell biology studies. This chromatin modification exists in undamaged cells, but chromatin restructuring adjacent to the DSB lesion was suggested to unmask the methylated residues to mediate the interaction (Huyen et al., 2004). In 2006, Botuyan et al. found that both 53BP1 and the putative fission yeast homolog Crb2 directly bind histone H4 specifically dimethylated at K20 (H4-K20me₂) using x-ray crystallography and NMR spectroscopy. Down-regulation of the enzyme Dot1 responsible for H3-K79 methylation was found to have no impact on 53BP1 accumulation at IR induced DSBs, whereas down-regulation of PR-Set7/Set8 responsible for H4-K20 monomethylation impaired the foci formation (Botuyan et al., 2006).

DSB resection

In the second pathway of the cellular response to DSBs, MRN and ATM also essentially initiate DSB resection and formation of single stranded DNA, which is essential for DNA repair by homologues recombination and ATR-dependent signaling (Jazayeri et al., 2005). The resection of the DSB requires activity of cdk's and is therefore restricted to S and G2 phases of the cell cycle (Jazayeri et al., 2005). Upon resection of DNA, the single stranded DNA is coated and stabilized by

RPA which facilitates the recruitment of the ATR-ATRIP complex (Cortez et al., 2001; Zou and Elledge, 2003). TopBP1 is needed to reach full activation of ATR and is recruited to the ssDNA independently of ATR/ATRIP (Kumagai et al., 2006; Mordes et al., 2008). ATR can then activate most of its substrates except Chk1 which requires the mediator protein Claspin (Liu et al., 2006). Chk1 is a very central checkpoint kinase and phosphorylates substrates involved in regulation of cell cycle progression, DNA repair and apoptosis. Alongside with checkpoint activation, repair of the DNA by HR is initiated by formation of Rad51-nucleofilament at the ssDNA to initiate strand invasion.

The second pathway is essentially also what is believed to happen upon detection of replication stress, but without resection. Upon stalling of DNA polymerases, the MCM replicative helicase is uncoupled from the polymerase and continue DNA unwinding ahead of the replication fork, generating ssDNA which binds RPA and recruits ATR through ATRIP (Byun et al., 2005). Upon replication block, the clamp loader Rad17 is furthermore recruited to the ssDNA and activated from phosphorylation by ATR (Bao et al., 2001) which then loads the Rad9-Rad1-Hus1 (911) complex onto DNA (Ellison and Stillman, 2003). The assembly of Claspin at the ssDNA upon replication stress requires activation of Rad17 (Wang et al., 2006), but it is not known yet, if this is the case after resection of DSBs.

Claspin was originally identified as a Chk1-binding protein in *Xenopus* egg extracts and found to be involved in regulating Chk1 activity (Kumagai and Dunphy, 2000). Human Claspin is nuclear and cell cycle regulated with peak in S/G2 and only associates with Chk1 upon replication stress or other types of DNA damage. Claspin becomes phosphorylated upon replication stress which is required for its association with Chk1 and the interaction promotes Chk1 phosphorylation by ATR. Down-regulation of Claspin with siRNA inhibited Chk1 activation upon replication stress, abrogated checkpoints and decreased cell survival. Claspin was therefore suggested to have an important role in replication checkpoint control (Chini and Chen, 2003). *Xenopus* Claspin associates with replication forks upon origin unwinding and is found to associate with Cdc45, DNA polymerase ϵ , RPA and two replication factor C complexes on chromatin. Only transient interaction with replication forks potentiates Chk1 activation and the stable binding of Claspin to chromatin is suggested to have other roles (Lee et al., 2005).

Spatial organization of the DDR proteins at DSBs

The proteins involved in the DNA damage response to DSBs interact with the site of damage in a carefully orchestrated manner. The interaction is both timely and spatially regulated, required to achieve efficient checkpoint signaling and repair. The nuclear rearrangement of chromatin in response to IR can be observed upon formation of so-called IR-induced foci (IRIF). IRIF are dynamic, microscopic structures with thousands of copies of protein involved in various aspect of DSB repair, and are used as a convenient mark of DSB location (Bekker-Jensen et al., 2006). Proteins associated with IRIF participate in various repair-associated DNA transactions, but also in restructuring large segments of chromatin in the vicinity of the DNA lesions. This furthermore allows access of DNA repair factors to damaged DNA.

By investigating recruitment of proteins to IRIF and studying their spatial pattern, two distinct regions of the IRIF can be distinguished which are the single stranded DNA (ssDNA) microcompartments and the DSB-flanking chromatin. Proteins interacting with the DSB-flanking chromatin assemble within the entire regions of modified chromatin that surrounds the DNA breaks and spans up to a megabase distance from the initial DSB lesion (Figure 11A). In the ssDNA microcompartment proteins assemble in much smaller areas called microfoci (Figure 11B). Unlike

the chromatin mediated interactions, assembly of proteins in the ssDNA microcompartments is restricted to S and G2 due to DSB resection (Bekker-Jensen et al., 2006).

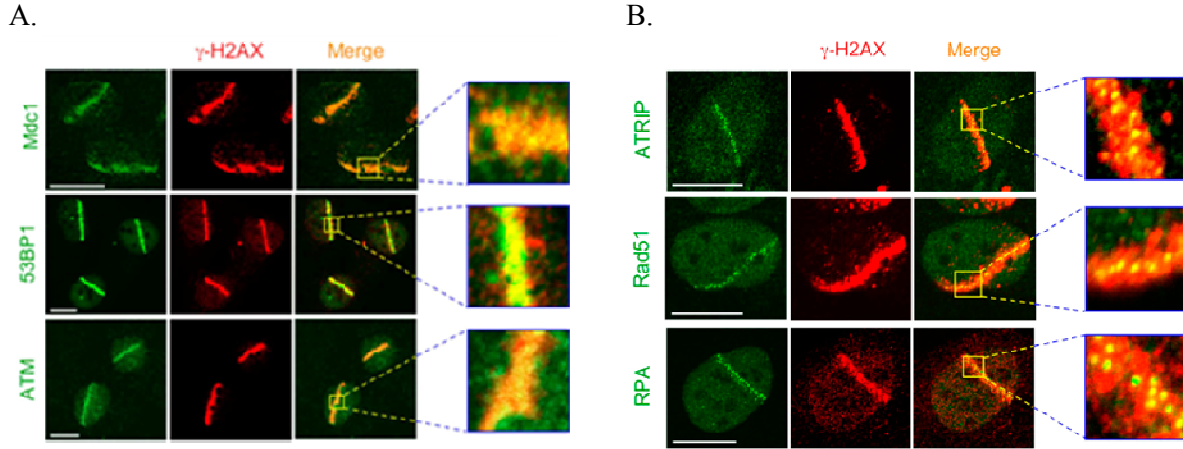


Figure 11: Spatial organization of DDR proteins at DSBs. (A) Proteins interacting with the DSB-flanking chromatin include Mdc1, 53BP1 and ATM. To visualize their interaction with chromatin, U2OS cells were sensitized with BrdU and microirradiated. 1 hour later, the cells were fixed and stained with antibodies against the indicated proteins and their co-localization with γ -H2AX show almost complete overlap (indicated by yellow from a merge of the two pictures). (B) Proteins interacting only with the subchromatin microfoci include Rad51, RPA and ATRIP. The cells were treated as in A. and from their merged pictures, only a slight overlap with γ -H2AX is observed which indicates the microfoci (Bekker-Jensen et al., 2006).

Proteins assembling at DSB-flanking chromatin include ATM, 53BP1 and Mdc1, whereas proteins assembling in microfoci include ATR, ATRIP, RPA, Rad51 and FANCD2. A striking feature of BRCA1 is to interact with both the DSB-flanking chromatin region throughout interphase and at microfoci in S and G2. Only the chromatin bound and not ssDNA associated retention of BRCA1 is controlled by Mdc1 (Bekker-Jensen et al., 2006). This can be observed in cells treated with siRNA against Mdc1. In these cells, BRCA1 only accumulates at microfoci and not at chromatin which is marked by γ -H2AX (Figure 12A and B). IRIF formation of 53BP1 on the other hand is completely dependent on Mdc1 and occurs only at the DSB-flanking chromatin. See Figure 12B.

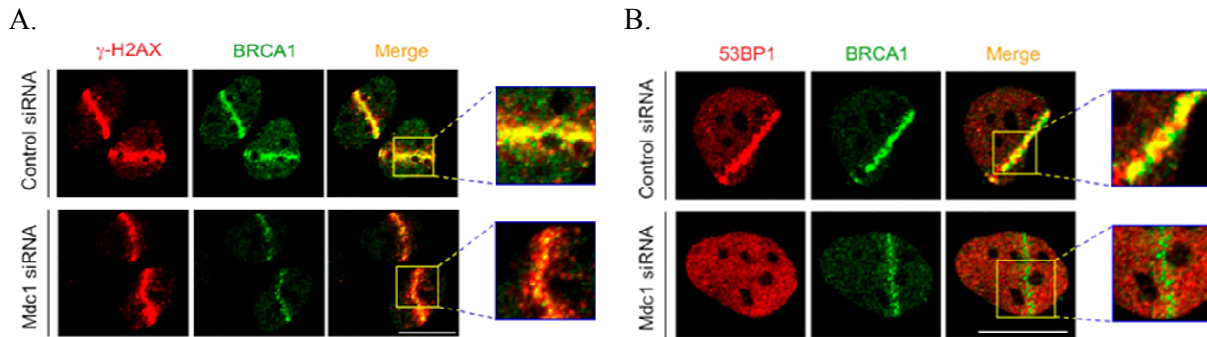


Figure 12: BRCA1 assembly at DSB-flanking chromatin and at microfoci. (A) To visualize the interaction of BRCA1 with chromatin, the cells were treated with siRNA as indicated before treatment as described in Figure 11A. The assembly of BRCA1 to DBS-flanking chromatin can be observed from the co-localization with γ -H2AX which is abrogated when treating the cells with siRNA against Mdc1. Only assembly at microfoci remains. (B) The cells were treated with siRNA as in A. before treatment described in Figure 11A. 53BP1 assembly is abrogated in Mdc1 siRNA treated cells in contrast to BRCA1 which still assembles at microfoci along the microirradiated track (Bekker-Jensen et al., 2006).

DNA damage checkpoints

DNA damage checkpoints are activated upon exposure to genotoxic insults which affects completion of a cell cycle phase. The pathways, which are also described above, include sensor proteins that monitor the presence of abnormalities in the DNA and help signaling to signal transducers and mediators which amplify and propagate the signal to downstream effectors that connect the checkpoint with the core cell cycle machinery. Different pathways are activated depending on the type of DNA damage and in which cell cycle phase the cell is in when the damage occur (Lukas et al., 2004).

G1/S

To prevent entering S-phase with damaged DNA and subsequent propagation of potentially hazardous mutations, cells facing DNA damage in G1 activate the checkpoint transducing kinases ATM/ATR and Chk1/Chk2 to delay or arrest cell cycle progression through two distinct pathways. The first involves the Cdc25A phosphatase and the second involves the transcription factor p53 (Bartek and Lukas, 2001; Lukas et al., 2004). Phosphorylation of Cdc25A and p53 by Chk1/Chk2 occurs rapidly and simultaneously, but the effect of the Cdc25A pathway is fast compared to the effect of the p53 pathway. Phosphorylation of Cdc25A mediates its ubiquitylation by SCF^{βTrCP} and degradation by the proteasome, which prevents activating dephosphorylation of Cdk2 and eventually prevents initiation of DNA synthesis (Busino et al., 2003). The Cdc25A pathway is relatively transient and p53 is necessary to achieve a sustained arrest in G1. p53 is phosphorylated by Chk1/Chk2 but also directly by ATM/ATR which furthermore targets the p53 ubiquitin ligase Mdm2. Collectively, this allows stabilization, accumulation and activation of p53 resulting in transcription of p53 targets including p21 which inhibits cdks. The fast and transient Cdc25A pathway is hereby replaced by the p53 pathway to keep a prolonged G1 arrest (Bartek and Lukas, 2001; Lukas et al., 2004).

Intra S

The checkpoint induced upon genotoxic stress during DNA replication causes only transient and reversible delay of the cell cycle progression. The intra-S checkpoint mainly inhibits initiation of unfired origins and thereby slows down replication. If the damage is not repaired during this delay, the cells will continue S phase, but with slower rate, and arrest later when reaching the G2/M checkpoint. The checkpoint operates mainly through the Cdc25A pathway as described above and is independent on p53. A multitude of other proteins are found to be involved and the exact mechanism of the checkpoint remains obscure (Bartek et al., 2004; Lukas et al., 2004).

G2/M

The G2/M checkpoint prevents cells from entering mitosis in the presence of damaged DNA. The checkpoint is activated both upon DNA damage encountered during G2 but also damage sustained from S phase. The key downstream target of this checkpoint is major mitosis promoting activity of Cyclin B-Cdk1 (Lukas et al., 2004; van Vugt and Medema, 2005). The checkpoint kinases ATM/ATR activates Chk1 and Chk2 which results in inactivation of all three Cdc25 phosphatases (A, B and C) that normally activates Cdk1 at entry into mitosis. The phosphatases are inactivated either through inhibitory phosphorylation or via ubiquitin-mediated degradation (Donzelli and Draetta, 2003). Chk1 has been proposed to phosphorylate Cdc25C at Ser216 to prevent its activation by sequestering Cdc25C in the cytoplasm through binding to 14-3-3 proteins (Sanchez et al., 1997; Peng et al., 1997). Also Cdc25B has been suggested to bind 14-3-3 upon phosphorylation by p38 *in vitro* to restrict Cdc25B to the cytoplasm (Bulavin et al., 2001). A subset of Chk1 has furthermore been suggested to localize at interphase centrosomes and appears to shield centrosomal Cdk1 from unscheduled activation by Cdc25B to restrain mitotic onset (Krämer et al., 2004). Phosphorylation of Cdc25A by Chk1 triggers SCF^{βTrCP} mediated ubiquitylation of Cdc25A followed

by rapid proteasomal degradation as described above. The degradation of Cdc25A together with sequestering Cdc25B and Cdc25C away keeps Cyclin B-Cdk1 in its inactive state thus blocking the G2/M transition. In *Xenopus*, Chk1 furthermore inhibits the cell cycle progression by activating Wee1, which is a negative regulator of Cdk1 (Lee et al., 2001). Also Plk1 was shown to be a direct target of the G2/M checkpoint (Smits et al., 2000a) and its catalytic inhibition was shown to be dependent on ATM/ATR (van Vugt et al., 2001). Furthermore, the phosphatase Cdc14B is suggested to translocate from the nucleolus to the nucleoplasm, upon DNA damage encountered in G2, and induce activation of APC^{Cdh1} which degrades Plk1. Degradation of Plk1 stabilizes Claspin and Wee1 to allow efficient G2/M checkpoint (Bassermann et al., 2008). Since Claspin is also a target of APC^{Cdh1} which has been shown in G0 and G1, USP28 is suggested to stabilize Claspin from APC^{Cdh1} mediated degradation in response to DNA damage (Bassermann et al., 2008). In addition, p53 is involved in the G2/M arrest by activating transcription of p21 which blocks the activating phosphorylation of Cdk1 on Thr161. This p53 mediated arrest is like in the G1/S arrest slower and is proposed to achieve a sustained G2 arrest in response to DNA damage (Smits et al., 2000b).

Recovery from G2/M

In *Xenopus* egg extracts with aphidicolin-induced (polymerase α inhibitor) DNA replication blocks, Claspin becomes phosphorylated on Thr906, which promotes its interaction with and phosphorylation of Ser934 by Plx1, a *Xenopus* Polo-like kinase. After prolonged interphase arrest, the extracts typically undergo adaptation and enter mitosis despite the presence of incompletely repaired DNA, resulting in a dissociation of Claspin from chromatin and inactivation of Chk1. Experiments with Claspin mutants unable to be phosphorylated, Claspin accumulated at chromatin and Chk1 activation was sustained and the extracts were not able to undergo adaptation. Plx1 was therefore suggested to be involved in terminating of a replication checkpoint response by inactivating Claspin (Yoo et al., 2004). Indeed, Plk1 was found to be critically required for recovery from a G2 checkpoint arrest in mammalian cells (van Vugt et al., 2004). Yet Claspin dissociation from chromatin in *Xenopus* is altered to Claspin degradation in mammalian cells as described below.

At the onset of mitosis in human cells, Claspin becomes degraded. Plk1 was found to prime Claspin for degradation by phosphorylating Ser30 and Ser34 of Claspin, leading to its recognition and ubiquitylation by SCF ^{β TrCP}. The degradation of Claspin was found to be dependent on an N-terminal β TrCP binding motif, DSGXXS. Impaired phosphorylation of the binding motif or down-regulation of β TrCP stabilized Claspin in mitosis, impaired Chk1 dephosphorylation and delayed entry into mitosis during recovery from cell cycle arrest imposed by DNA damage or replication stress (Mailand et al., 2006; Mamely et al., 2006; Peschiaroli et al., 2006). Undegraded Claspin in cells after passing the G2/M checkpoint allowed partial reactivation of Chk1 in cells exposed to DNA damage. Degradation of Claspin was therefore suggested to facilitate timely reversal of the checkpoint response and define the period permissive for Chk1 activation during cell cycle progression (Mailand et al., 2006).

Degradation of Claspin accumulates Cdc25A due to inactivation of Chk1 and Cdc25A then dephosphorylates and activates Cdk1 and Cdk2. The increase in Cdk1 activity is suggested to phosphorylate Wee1 to prime Wee1 for Plk1 phosphorylation which recruits SCF ^{β TrCP} and results in ubiquitylation and degradation. This furthermore increases the Cdk1 activity to allow cells to resume cell cycle progression and enter mitosis (Watanabe et al., 2004). An overview of checkpoint initiation and recovery can be observed in Figure 13. Interestingly SCF ^{β TrCP} function as a molecular switch, from Cdc25A degradation upon checkpoint initiation to Claspin and Wee1 degradation upon checkpoint recovery. It appears that it is the phosphorylation state of Cdc25A, Claspin and Wee1 that allows SCF ^{β TrCP} to change its target (Bartek and Lukas, 2007).

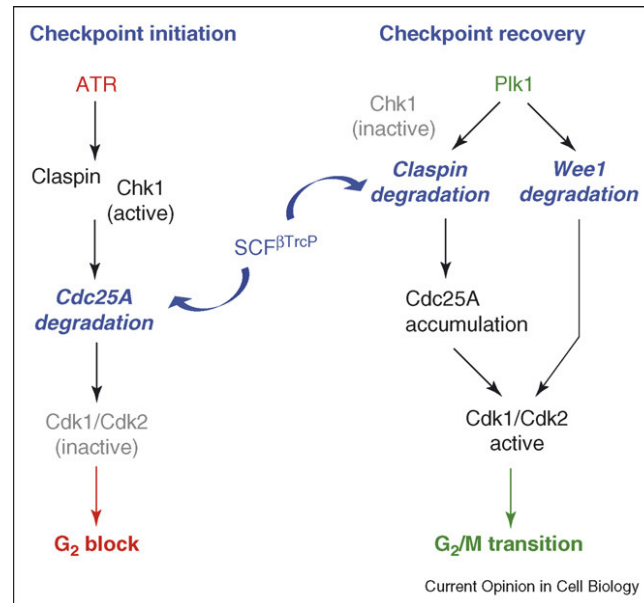


Figure 13: Checkpoint initiation and recovery. Upon checkpoint initiation, ATR becomes activated and phosphorylates Chk1 in a Claspin dependent manner. Chk1 phosphorylation of Cdc25A primes the phosphatase for degradation by the SCF^{βTrCP} ubiquitin ligase which keeps Cdk1 and Cdk2 in their inactive phosphorylated state which arrest the cells in G₂. Upon checkpoint recovery when the damage is repaired, Plk1 phosphorylates Claspin which targets the protein for degradation by the SCF^{βTrCP} complex. The increase in the phosphatase activity of Cdc25A together with lower inhibiting phosphorylation activity of Wee1, which is also targeted for degradation allows activation of Cdk1 and Cdk2, and the cell can continue cell cycle progression and enter mitosis (Bartek and Lukas, 2007).

4 Results and Discussion

Upon exposure to genotoxic agents, efficient repair of DNA damage is coordinated with the cell cycle to ensure low occurrence of mutations, chromosomal translocations and chromosome loss, to preserve the integrity of the genome. The aim of this PhD thesis is to increase our knowledge on how cells apply ubiquitylation to co-ordinate these responses to DNA damage efficiently.

In the Results and Discussion part, the two main projects, I have been working on during my PhD, are presented. Each project will be presented separately and includes the original publication or manuscript in press, followed by a discussion which relates the obtained data to earlier and later findings. The supplemental data of each publication is given in the appendix.

In the first project, the novel role of the RNF8 ubiquitin ligase in the DNA damage response is presented. RNF8 was found to accumulate at DSB-flanking chromatin upon DNA damage through binding to Mdc1 and ubiquitylate histones to facilitate recruitment of 53BP1 and BRCA1 to sites of damage. In the second project, the novel role of the deubiquitylating enzyme USP7 in maintaining steady-state levels of Claspin is presented. Claspin was furthermore found to be degraded by APC^{Cdh1} in G1 and USP7 was found to oppose the SCF^{βTrCP}- but not the APC^{Cdh1}-mediated degradation of Claspin.

4.1 Publication 1

Niels Mailand, Simon Bekker-Jensen, **Helene Faustrup**, Fredrik Melander, Jiri Bartek, Claudia Lukas and Jiri Lukas. RNF8 Ubiquitylates Histones at DNA Double-Strand Breaks and Promotes Assembly of Repair Proteins. *Cell* 131, 887-900, 2007.

RNF8 Ubiquitylates Histones at DNA Double-Strand Breaks and Promotes Assembly of Repair Proteins

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DOI 10.1016/j.cell.2007.09.040

SUMMARY

Accumulation of repair proteins on damaged chromosomes is required to restore genomic integrity. However, the mechanisms of protein retention at the most destructive chromosomal lesions, the DNA double-strand breaks (DSBs), are poorly understood. We show that RNF8, a RING-finger ubiquitin ligase, rapidly assembles at DSBs via interaction of its FHA domain with the phosphorylated adaptor protein MDC1. This is accompanied by an increase in DSB-associated ubiquitylations and followed by accumulation of 53BP1 and BRCA1 repair proteins. Knockdown of RNF8 or disruption of its FHA or RING domains impaired DSB-associated ubiquitylation and inhibited retention of 53BP1 and BRCA1 at the DSB sites. In addition, we show that RNF8 can ubiquitylate histone H2A and H2AX, and that its depletion sensitizes cells to ionizing radiation. These data suggest that MDC1-mediated and RNF8-executed histone ubiquitylation protects genome integrity by licensing the DSB-flanking chromatin to concentrate repair factors near the DNA lesions.

INTRODUCTION

Among the most prominent cytological manifestations of DNA breakage is formation of the ionizing radiation-induced foci (IRIF). These nuclear structures reflect local chromatin expansion, posttranslational modifications of histones, and assembly of diverse proteins in the vicinity of chromosomal lesions (Fernandez-Capetillo et al., 2004; Kruhlak et al., 2006). In mammals, phosphorylation of histone H2AX (γ -H2AX) by the ATM kinase is among the most proximal DSB-induced histone modifications and the prerequisite for the sustained retention of signaling

and repair proteins. Specifically, the proteins that avidly accumulate on the γ -H2AX-marked chromatin include the components of the MRE11-NBS1-RAD50 (MRN) complex, the ATM kinase, and a group of large adaptor proteins such as MDC1, 53BP1, and BRCA1 (Bekker-Jensen et al., 2006). The increased concentration of these proteins is believed to enhance the effectiveness of genome surveillance mechanisms by amplifying ATM signaling and increasing the efficiency of DSB repair (Lukas et al., 2004b).

The assembly of proteins at the DSB-flanking chromatin proceeds with distinct temporal patterns, suggesting that the formation of the DSB-induced chromatin microenvironment is a hierarchical and highly regulated process (Bekker-Jensen et al., 2005; Lukas et al., 2004a). The key interaction module engaged in direct binding to γ -H2AX resides in the BRCT domains of MDC1 (Stucki et al., 2005). MDC1 is among the first proteins to accumulate at the DSB sites (Lukas et al., 2004a), and the assembly of the γ -H2AX-MDC1 complex seems to be required for retention of most other proteins in these regions (Stucki and Jackson, 2006). Furthermore, MDC1 binding shields the C-terminal tail of γ -H2AX against premature dephosphorylation (Stewart et al., 2003; Stucki et al., 2005), a feature that helps maintain a favorable chromatin configuration until the completion of the DSB repair. Thus, MDC1 emerges as the “master regulator” determining the formation of a specific chromatin microenvironment required for genomic stability.

The way MDC1 promotes chromatin retention of other proteins involves at least two distinct mechanisms. First, there is biochemical evidence that the retention of NBS1 at the DSB-flanking chromatin is mediated by its direct interaction with MDC1 (Goldberg et al., 2003; Lukas et al., 2004a; Stewart et al., 2003). Real-time imaging of living cells supports this model by showing that MDC1 and NBS1 accumulate at the DSB-flanking chromatin very rapidly and with the same kinetics (Lukas et al., 2004a). Because NBS1 also directly binds ATM (Falck et al., 2005; You et al., 2005), the MDC1-mediated retention of NBS1 has a crucial physiological significance for the initial

stages of the DSB response as it promotes spreading of γ -H2AX from the initial breakage sites and facilitates other ATM-dependent phosphorylations (Stucki and Jackson, 2006). Second, although the retention of 53BP1 at the DSB sites is also dependent on MDC1, its accumulation at the DSB-flanking chromatin is significantly delayed (Bekker-Jensen et al., 2005). Based on this observation, and the fact that 53BP1 interacts with DSBs via binding to constitutively methylated residues on histones (Botuyan et al., 2006; Huyen et al., 2004), it has been proposed that MDC1 promotes higher-order chromatin restructuring that may increase the accessibility of histone marks. Alternatively, the formation of the γ -H2AX-MDC1 complex may promote additional modifications of the DSB-flanking chromatin that in conjunction with methylated histones increase the affinity for 53BP1.

Although intriguing, our understanding of the dynamics (and indeed the purpose) of the DSB-induced chromatin response is only rudimentary, and the above concepts leave several unanswered questions. Most notably, although it has been shown that the MDC1-NBS1 interaction requires the intact FHA domain of the latter protein (Lukas et al., 2004a), the exact structural requirements for this interaction are not understood, and whether other proteins accumulate at the DSB sites via direct binding to MDC1 has not been determined. Even more elusive are the structural and/or posttranslational events that operate between the rapid assembly of the γ -H2AX-MDC1 complexes and the delayed accumulation of 53BP1. Finally, chromatin retention of BRCA1 is also regulated by MDC1 (Bekker-Jensen et al., 2006; Lou et al., 2003) and requires at least two additional proteins, RAP80 and ABRA1 (Kim et al., 2007; Sobhian et al., 2007; Wang et al., 2007; Yan et al., 2007). However, how MDC1 functionally interacts with the RAP80-ABRA1 complex to regulate chromatin retention of BRCA1 remains an outstanding puzzle in the biology of this important tumor suppressor. In this study, we attempted to resolve these issues.

RESULTS

RNF8 Is a Novel DSB Regulator, which Binds to Chromatin in an MDC1-Dependent Manner

We performed a bioinformatic search for proteins that contain structural motifs shared by the established DSB regulators and tested their ability to accumulate at the DSB-modified chromatin. By this approach, we identified RNF8, a member of the RING-finger-containing nuclear factors (Ito et al., 2001; Plans et al., 2006), which contains a phosphothreonine-binding FHA domain at its N terminus and a RING-finger domain at the C terminus (Figure 1A). Both domains have been previously identified in chromatin-associated DSB regulators such as NBS1 and MDC1 (the FHA domain) (Stracker et al., 2004; Stucki and Jackson, 2006) or the BRCA1-BARD1 complex (the RING domain) (Boulton, 2006). Importantly, both green fluorescence protein (GFP)-tagged RNF8 (Figures S1A and S1B) and endogenous RNF8 avidly accumulated in IRIF and in

microlaser-generated DSB tracks, respectively (Figures 1B–1E), and several pieces of evidence indicated that this DNA damage-induced accumulation reflected targeting of RNF8 to the DSB-flanking chromatin. Thus, GFP-RNF8 spanned the entire chromatin regions marked by γ -H2AX (Figure 1C, left). In addition, GFP-RNF8 accumulation at the DSBs was equally robust throughout interphase (Figure 1C, right), a feature shared by all known components of the DSB-flanking chromatin (Bekker-Jensen et al., 2006). But most significantly, the DSB-induced accumulation of RNF8 was abrogated after siRNA-mediated ablation of MDC1, the key upstream regulator of the DSB-induced chromatin response (Figure 1F). Together, these data suggest that RNF8 is a new DSB regulator that interacts with the DSB-flanking chromatin.

The FHA Domain of RNF8 Binds Phosphorylated MDC1

To elucidate how RNF8 interacts with the DSB sites, we asked whether it binds MDC1. Indeed, endogenous RNF8 and MDC1 interacted in a coimmunoprecipitation assay (Figure 2A), and we set out to explore the structural basis of this interaction. While the wild-type (WT) and RING-deficient (C403S; *RING) versions of RNF8 bound MDC1 (Figure 2B), a point mutation of a conserved residue within the FHA domain of RNF8 (R42A; *FHA) abolished the interaction (Figure 2B). Consistently, the GFP-tagged *FHA version of RNF8 failed to form nuclear foci after ionizing radiation (IR), whereas the wild-type protein or the *RING mutant avidly accumulated at DSBs (Figure 2C). We then performed a deletion analysis of the MDC1 N terminus and found that the region between amino acids 698 and 800 was necessary and sufficient for RNF8 binding (Figure S2A). This region of MDC1 contains three conserved T-Q-X-F clusters (Figure S2B) fulfilling the criteria for ATM/ATR substrates (Shiloh, 2003). While single or double substitutions of the threonine residues with alanines had little effect on binding of MDC1 to RNF8 (our unpublished data), simultaneous threonine mutations in all three clusters (designated as 3A) completely abolished this interaction (Figure 2D). To validate that the T-Q-X-F clusters could be phosphorylated *in vivo*, we exposed cells expressing the wild-type or the 3A fragments of MDC1 to IR and analyzed the immunopurified MDC1 proteins by immunoblotting with an antibody to phosphorylated SQ/TQ motifs (the consensus sites for ATM/ATR targets). Although wild-type MDC1 was phosphorylated to some extent even in nonirradiated cells (likely due to the low intrinsic ATM/ATR activity), this phosphorylation markedly increased after exposing the cells to IR, and mutation of the T-Q-X-F clusters impaired both the basal and the IR-induced phosphorylation of MDC1 (Figure 2E). Consistently, while FLAG-RNF8 and endogenous MDC1 interacted to some extent in unstressed cells, this interaction was enhanced after IR (Figure 2F). Together, these data suggest that phosphorylation of the conserved T-Q-X-F clusters of MDC1 and the integrity of the FHA domain of RNF8 are required to mediate the interaction between these two proteins.

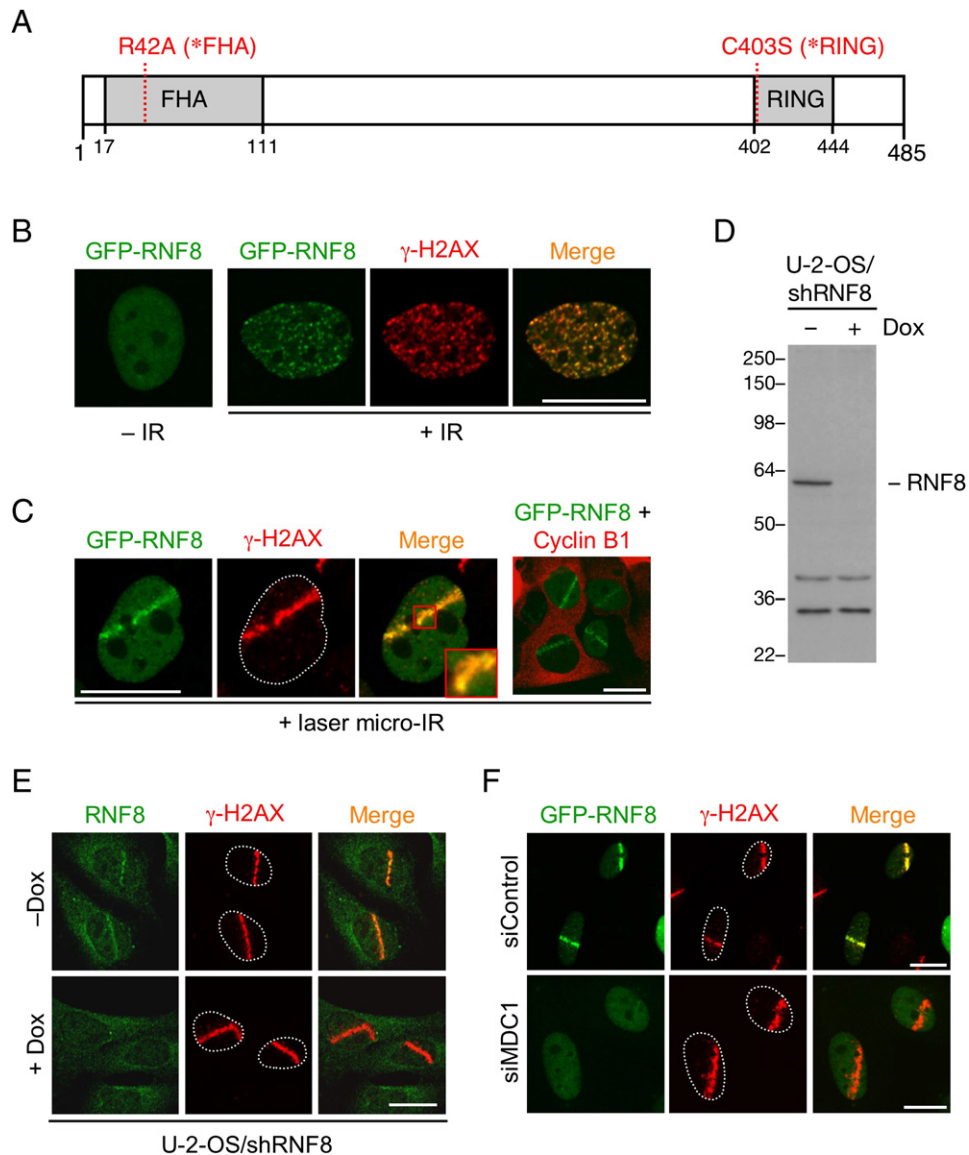


Figure 1. Accumulation of RNF8 at the DSB Sites Is Regulated by MDC1

(A) Schematic structure of human RNF8. Positions of residues mutated to generate RNF8 *FHA (R42A) and RNF8 *RING (C403S) are indicated.

(B) U-2-OS cells stably expressing GFP-RNF8 (U-2-OS/GFP-RNF8) before (left) and 30 min after (right) exposure to IR (4 Gy) were immunostained for γ -H2AX.

(C) U-2-OS/GFP-RNF8 cells were microirradiated and after 30 min immunostained for γ -H2AX (left) or for cyclin B1 (right). RNF8 accumulates at laser tracks regardless of cyclin B1, indicating that its interaction with the DSB sites is cell cycle independent.

(D) U-2-OS cells conditionally expressing shRNA to RNF8 (U-2-OS/shRNF8) cells were induced or not with Doxycycline (Dox) for 48 hr and analyzed by immunoblotting with a rabbit antibody to RNF8.

(E) U-2-OS/shRNF8 cells were induced or not with Dox for 48 hr, exposed to laser microirradiation, and 1 hr later coimmunostained with antibodies to RNF8 and γ -H2AX. WCE, whole-cell extract; scale bar, 10 μ m.

(F) U-2-OS/GFP-RNF8 cells were transfected with the indicated siRNA oligonucleotides for 96 hr, microirradiated as in (C), and immunostained for γ -H2AX. Scale bars, 10 μ m.

Phosphorylation of MDC1 Determines Retention of RNF8 at the DSB Sites

To test the significance of the MDC1-RNF8 interaction directly in cells, we downregulated endogenous MDC1 by shRNA (Bekker-Jensen et al., 2006) and reintroduced

into these cells either WT or the 3A version of MDC1 (both full-length and resistant to the shRNA) together with GFP-RNF8. As expected, the MDC1 knockdown abolished accumulation of GFP-RNF8 at the sites of DNA damage (Figure 2G, top). Strikingly, although MDC1-WT

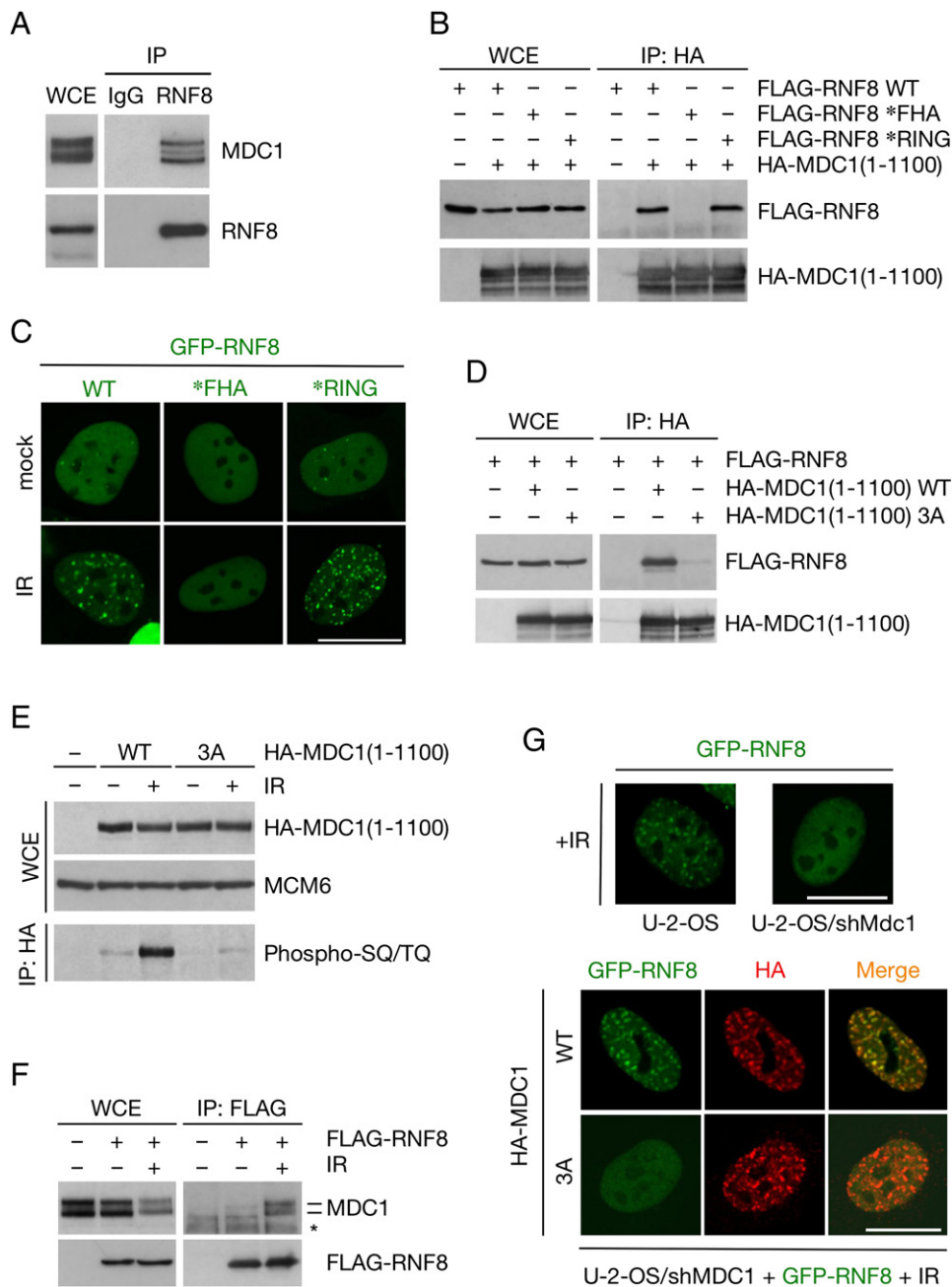


Figure 2. The FHA Domain of RNF8 Interacts with Phosphorylated MDC1

(A) Lysates from HEK293T cells were immunoprecipitated with control or RNF8-specific antibodies followed by immunoblotting with antibody to MDC1.

(B) HEK293T cells were cotransfected with the HA-tagged N-terminal fragment of MDC1 (amino acids 1–1100) and the indicated versions of FLAG-RNF8. Interactions between MDC1 and RNF8 were assessed by immunoprecipitation (IP) of HA-tagged proteins followed by immunoblotting with anti-FLAG antibody.

(C) U-2-OS cells stably expressing wild-type (WT), FHA-deficient (*FHA), or RING-deficient (*RING) forms of GFP-RNF8 were left untreated (top) or exposed to 4 Gy of IR (bottom). Images were acquired after 30 min.

(D) HEK293T cells were cotransfected with indicated combinations of plasmids, and association between RNF8 and MDC1 was analyzed by coimmunoprecipitation as in (A).

(E) HEK293T cells transfected with HA-tagged WT or 3A fragments of MDC1 (1–1100) were exposed or not to IR (10 Gy). One hour later, the extent of MDC1 phosphorylation was assayed by immunoprecipitation with anti-HA antibody followed by immunoblotting with the phospho-SQ/TQ antibody. MCM6 is a loading control. WCE, whole-cell extract.

(F) U-2-OS cells were transfected with FLAG-RNF8 and exposed or not to IR (10 Gy). One hour later, binding between ectopic RNF8 and endogenous

and MDC1-3A were expressed to similar levels (Figure S3A) and formed readily discernible IR-induced foci (Figure 2G, bottom), only the WT protein was able to restore GFP-RNF8 accumulation at the DSB sites (Figure 2G, bottom). Interestingly, while mutation of the T-Q-X-F clusters completely uncoupled MDC1 from RNF8 (Figures 2D and 2G), the same MDC1-3A mutant remained fully proficient to interact with NBS1 (Figure S3B) and support NBS1 focus formation (Figure S3C). Because NBS1 also contains an FHA domain, which is required for its interaction with MDC1 and accumulation at the DSB sites (Lukas et al., 2004a), these data support the emerging concept of MDC1 as a molecular matchmaker capable of organizing multiple signaling and repair factors in the vicinity of DNA breaks and suggest that the main purpose of the ATM-mediated phosphorylation of the T-Q-X-F clusters is to recruit RNF8.

RNF8 Rapidly Assembles at the DSB-Flanking Chromatin

To investigate the function of RNF8 at the sites of DNA damage, we applied a kinetic assay based on micro-laser-generated DSBs coupled with a real-time imaging of protein redistribution (Bekker-Jensen et al., 2006; Lukas et al., 2003). By this approach, we found that the accumulation of GFP-RNF8 in the microirradiated regions was extremely rapid: the first signs of local concentration of GFP-RNF8 were detectable in less than a minute, and the steady-state accumulation in the DSB areas was reached between 6 and 8 min after microirradiation (Figure 3A, top). We noticed that while the assembly kinetics of GFP-RNF8 was similar to that of GFP-MDC1 (Figure 3A, middle), it appeared faster than the accumulation of GFP-BRCA1 (Figures 3A, bottom and S1C). This prompted us to compare the kinetic parameters of all core components of the DSB-flanking chromatin under strictly identical conditions (Bekker-Jensen et al., 2005, 2006; Lukas et al., 2004a). A quantitative analysis of the fluorescence intensities of MDC1, NBS1, 53BP1, BRCA1, and RNF8 (all GFP-tagged, stably expressed in an isogenic parental cell line, and tested for their functionality) revealed that their accumulation at the DSB-flanking chromatin clusters into two distinct kinetic groups: the “early” group comprising MDC1, NBS1, and RNF8, followed by the “late” group including 53BP1 and BRCA1 (Figure 3B). The delayed assembly of BRCA1 was confirmed at the level of endogenous proteins by comparing its accumulation with that of 53BP1, the known late component of the DSB-modified chromatin (Bekker-Jensen et al., 2005) (Figure S4A). Conversely, we found that endogenous RNF8 accumulated at the DSB sites as early as MDC1,

the most upstream protein to arrive at the γ -H2AX-marked chromatin (Figure S4B). Finally, the assembly of ATR, a protein that specifically interacts with single-stranded DNA generated by DSB resection (Cortez et al., 2001; Bekker-Jensen et al., 2006), was significantly delayed compared to both early and late chromatin assembly waves (Figure 3B), suggesting that the chromatin response in general precedes the DNA-end resection. Collectively, these results demonstrated that our experimental conditions were sensitive enough to temporally dissect the DSB-induced chromatin formation, and that RNF8 is among the first proteins to assemble in this compartment.

A Ubiquitin-Regulated Event Couples the Rapid and Delayed Accumulation of Proteins at the DSB-Modified Chromatin

The finding that the accumulation of 53BP1 and BRCA1 at DSBs was delayed, dependent on MDC1 (Bekker-Jensen et al., 2005, 2006; Lou et al., 2003) yet each independent of the other (Figure S5), suggested that both 53BP1 and BRCA1 share a common, MDC1-regulated denominator required for their productive retention in the DSB-flanking chromatin. Since RNF8 binds MDC1, accumulates rapidly at the DSB sites, and contains a RING domain typical for the E3 ubiquitin ligases (Ito et al., 2001; Plans et al., 2006), we reasoned that it, and its enzymatic activity, might couple the assembly of MDC1 with the ensuing accumulation of 53BP1 and BRCA1.

To test this hypothesis, we first asked whether regulatory ubiquitylation as such contributes to the accumulation of DSB regulators on the DNA damage-modified chromatin. We exploited the recent findings showing that inhibition of the 26S proteasome causes a rapid depletion of free nuclear ubiquitin due to accumulation of nondegraded polyubiquitylated proteins in the cytosol (Dantuma et al., 2006). Indeed, also in our cellular system proteasome inhibitors such as MG132 triggered progressive translocation of GFP-ubiquitin, as well as the ubiquitin-protein conjugates, from the nucleus to the cytoplasm, a process that was completed between 60 and 90 min after MG132 addition (Figure S6A). This was accompanied by a loss of monoubiquitylated histones (Figure S6B), confirming a severe impairment of regulatory ubiquitylations in the nucleus (Dantuma et al., 2006). Strikingly, such depletion of free nuclear ubiquitin had a very differential impact on the ability of proteins to accumulate at the DSB-flanking chromatin. When cells were pretreated with MG132 and then subjected to laser microirradiation, the pattern and extent of the γ -H2AX-decorated chromatin developed normally, and MDC1, NBS1, and RNF8 (the early

MDC1 was monitored by immunoprecipitation with anti-FLAG antibody followed by immunoblotting with anti-MDC1 antibody (the lower MDC1 signal in lane 3 is due to increased chromatin retention of MDC1 after DNA damage). Asterisk indicates a crossreacting band.

(G) U-2-OS cells, or their derivatives stably expressing the MDC1-targeting shRNA (U-2-OS/shMDC1), were transiently transfected with GFP-RNF8 and exposed to IR (4 Gy, 30 min). The ability of GFP-RNF8 to form IR-induced foci was monitored by confocal microscopy (top). U-2-OS/shMDC1 cells were cotransfected with GFP-RNF8 and the indicated versions of HA-tagged MDC1 (all in the context of full-length MDC1), exposed to IR (4 Gy, 30 min), and immunostained with anti-HA antibody (bottom). Scale bars, 10 μ m.

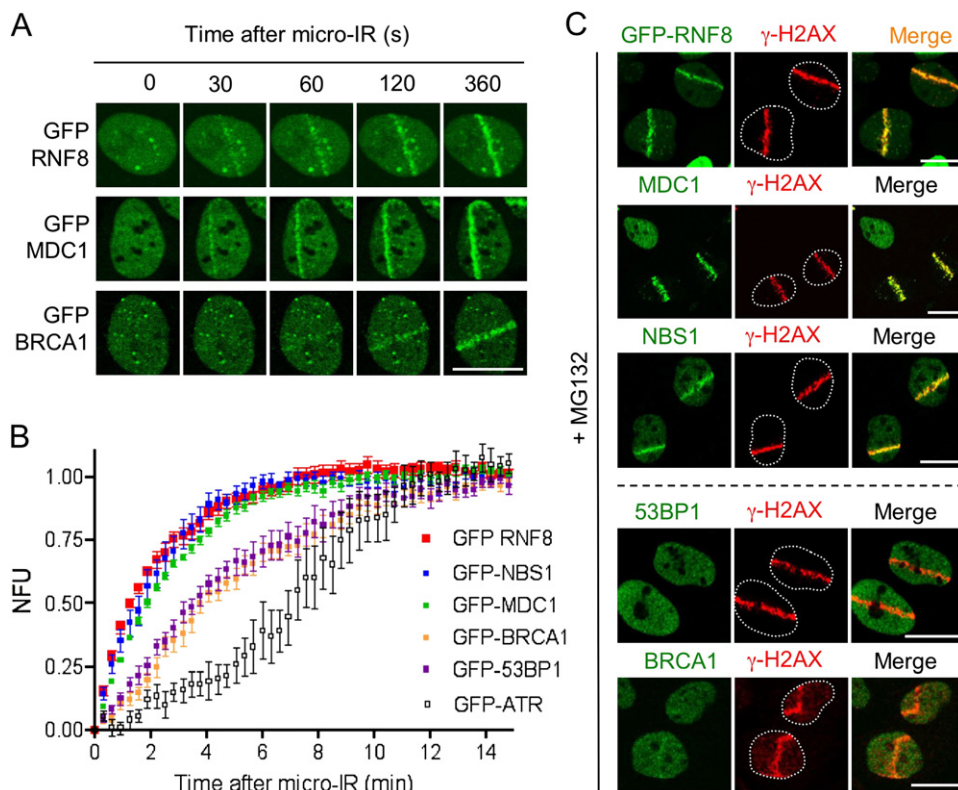


Figure 3. Protein Assembly at the DSB-Flanking Chromatin Follows Two Distinct Temporal Waves

(A) U-2-OS cell lines stably expressing the indicated GFP-tagged proteins were exposed to laser microirradiation followed by a real-time recording of the protein assembly kinetics.

(B) U-2-OS cell lines were assayed as in (A). The fluorescence intensity values in the microirradiated areas were pooled from 10 independent cells per each protein and plotted on a time scale. NFU, normalized fluorescence units. Error bars represent twice the standard error of the normalized data at the respective time points.

(C) U-2-OS/GFP-RNF8 cells (top) or the parental U-2-OS cells (the remaining panels) were incubated for 90 min with MG132, microirradiated by the laser, and after an additional 30 min immunostained with the indicated antibodies. Scale bars, 10 μ m.

kinetic group) accumulated at the DSB sites (Figure 3C, top panels). In contrast, the accumulation of BRCA1 and 53BP1 (the late kinetic group) was abrogated under these conditions (Figure 3C, bottom panels). Consistently, when assayed in cells that had been first microirradiated and only then challenged with MG132, 53BP1 (the late assembly group) but not NBS1 (the early assembly group) dissociated from the microirradiated compartments with the kinetics that mirrored the pace of the nuclear ubiquitin depletion (Figure S6A). Thus, while regulatory ubiquitylation is not required for the initial DSB-induced chromatin response, it appears essential for generating conditions permissive for the second wave of protein accumulation.

RNF8 Ubiquitylates the DSB-Flanking Chromatin and Promotes Accumulation of 53BP1 and BRCA1 in This Compartment

We then designed two independent RNF8-targeting siRNA oligonucleotides, each of which efficiently downregulated RNF8 (Figure S7A). Strikingly, RNF8 knockdown essentially recapitulated chemical depletion of the free

nuclear ubiquitin by allowing normal assembly of MDC1 and NBS1 (Figure 4A, left panels) but preventing accumulation of 53BP1 and BRCA1 at the DSB-flanking chromatin (Figure 4A, right panels). The residual BRCA1 retention in the microirradiated regions was not unexpected given that a fraction of BRCA1 directly binds DNA (Paull et al., 2001), and that this type of interaction is independent of chromatin (Bekker-Jensen et al., 2006). Larger magnification confirmed that the RNF8 knockdown reduced BRCA1 accumulation to small subchromatin “microfoci” typical for ssDNA-binding proteins (Figure 4A, insets). Independent experiments confirmed that downregulation of RNF8 did not prevent DSB resection and formation of the RPA-coated ssDNA compartments (Figure S7B).

Consistent with earlier studies reporting local ubiquitylation in the IR-induced nuclear foci (Morris and Solomon, 2004; Polanowska et al., 2006) we noticed that laser microirradiation induced local accumulation of conjugated ubiquitin (Figure 4B). Remarkably, this was drastically impaired both by depletion of nuclear ubiquitin (achieved by a short treatment with MG132 before microirradiation) and

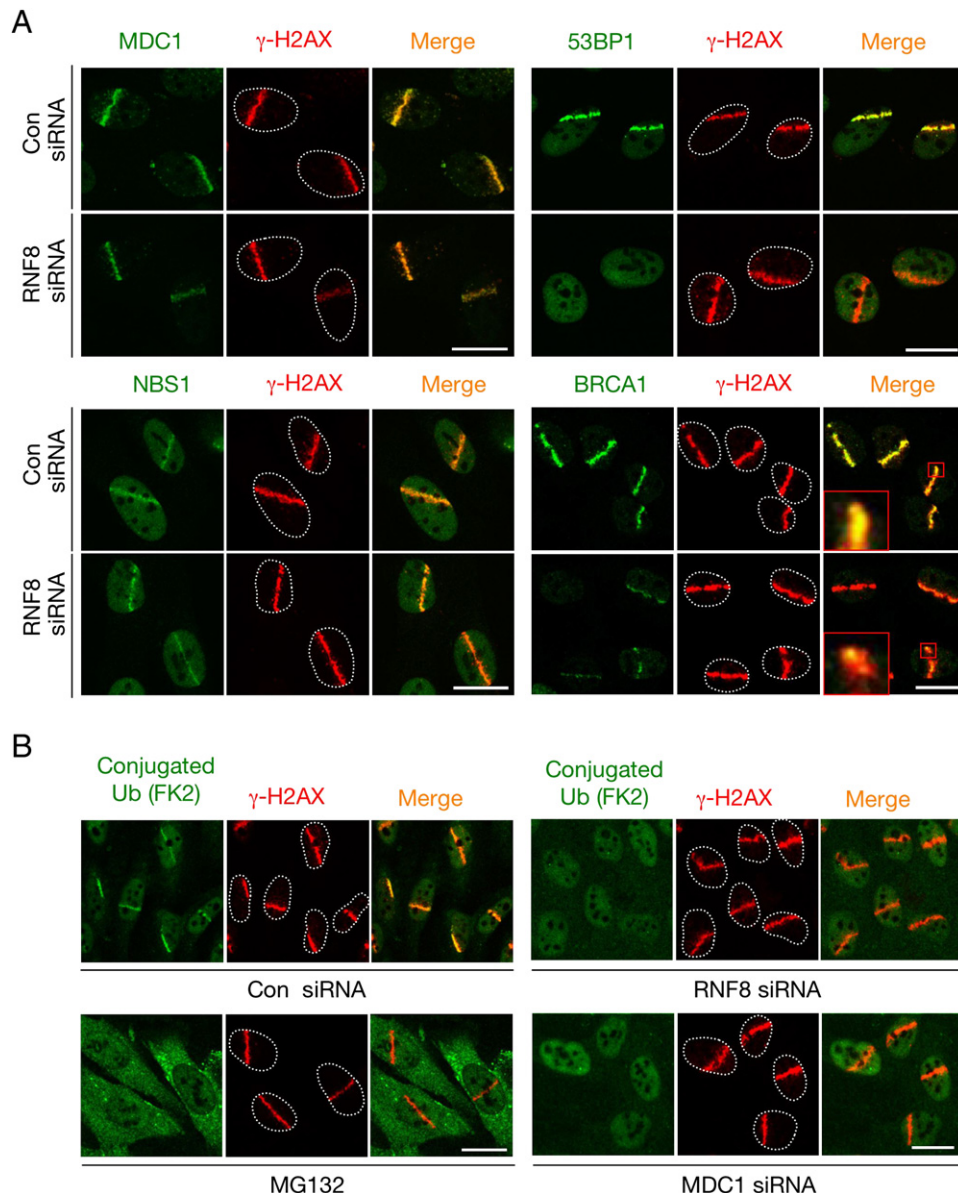


Figure 4. Knockdown of RNF8 Impairs Accumulation of 53BP1, BRCA1, and Conjugated Ubiquitin at the DSB-Flanking Chromatin
 (A) U-2-OS cells were transfected with control or RNF8-targeting siRNA oligonucleotides, microirradiated, and 1 hr later immunostained with the indicated antibodies.

(B) U-2-OS cells were transfected with the indicated siRNA oligonucleotides or treated for 90 min with MG132 as indicated, microirradiated, incubated for 1 hr, and coimmunostained with antibodies to conjugated ubiquitin (FK2) and γ -H2AX. Scale bars, 10 μ m.

by siRNA-mediated knockdown of RNF8 (Figure 4B). Although knockdown of BRCA1 also reduced the DSB-associated accumulation of conjugated ubiquitin as previously reported (Morris and Solomon, 2004; Polanowska et al., 2006), the effect of RNF8 was consistently much more dramatic (Figures 4B, S8A, and S8B) and comparable to that achieved by depletion of MDC1, the mediator of RNF8 recruitment to the DSB-flanking chromatin (Figure 4B). Together, these data suggest that the RNF8 ubiquitin ligase directly contributes to DSB-induced ubiquitylations that in

turn function as an important trigger for 53BP1 and BRCA1 retention in the neighboring chromatin. In support of this conclusion, knockdown of RNF8 abrogated DSB retention of RAP80 (Figure 5A), a ubiquitin-binding protein that is essential for BRCA1 recruitment to the DSB sites (see Introduction). Interestingly, RNF8 remained associated with the DSB sites as long as these were marked by γ -H2AX (Figure S4C), suggesting that its activity facilitates accumulation of BRCA1 and 53BP1 in the vicinity of DNA breaks until these are productively repaired.

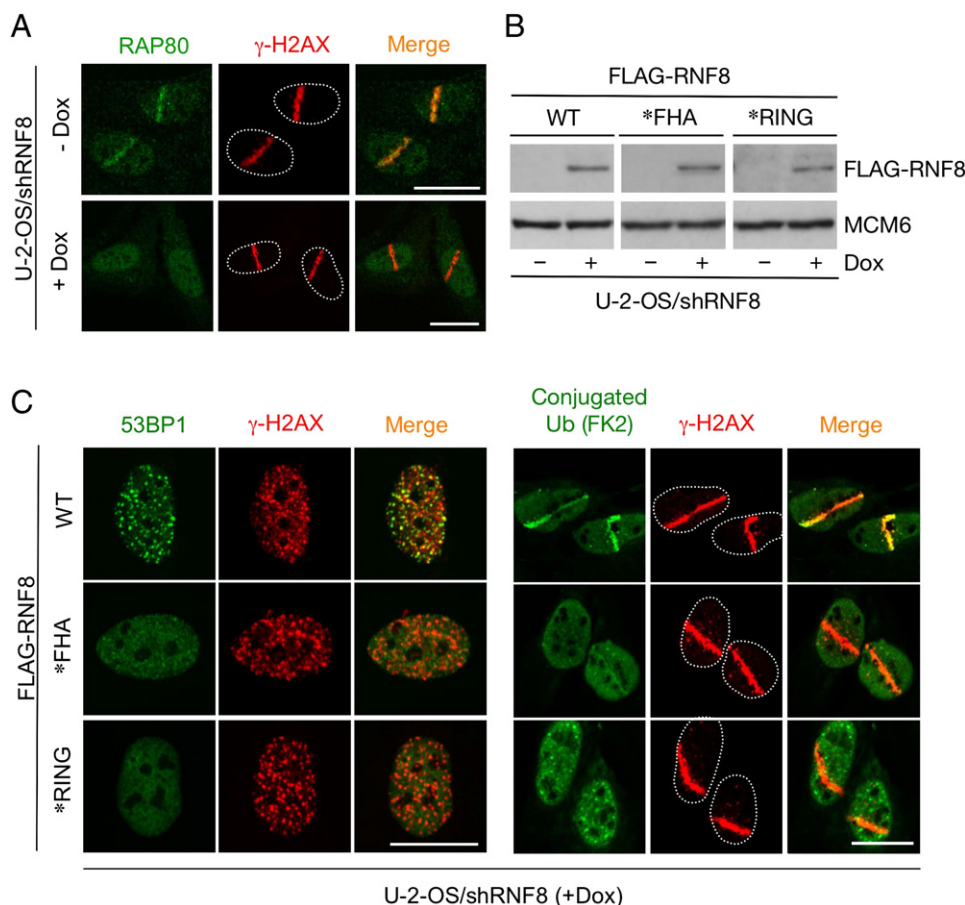


Figure 5. Local Concentration and Catalytic Activity of RNF8 Determine Retention of 53BP1 at the DSB-Flanking Chromatin

(A) U-2-OS/shRNF8 cells were induced or not with Dox for 48 hr, exposed to laser microirradiation, and 1 hr later coimmunostained with antibodies to RAP80 and γ -H2AX.

(B) U-2-OS/shRNF8 cells engineered to induce various alleles of FLAG-tagged, shRNA-resistant RNF8 were incubated or not with Dox, and the expression of the induced RNF8 proteins was monitored by immunoblotting with anti-FLAG antibody.

(C) U-2-OS/shRNF8/FLAG-RNF8 cells from (B) were induced with doxycycline (Dox) for 48 hr and exposed to IR (4 Gy) (left) or microirradiated by the laser (right). After 1 hr, the cells were coimmunostained with antibodies to γ -H2AX and 53BP1 (left) or conjugated ubiquitin (FK2) (right). Scale bars, 10 μ m.

To test the impact of RNF8 on the DSB-induced chromatin maturation in vivo, we generated cell lines allowing a conditional replacement of the endogenous RNF8 by the WT, *FHA, or *RING versions of RNF8 achieved by a doxycycline-dependent induction of the RNF8-targeting shRNA combined with a simultaneous induction of the respective RNF8 alleles resistant to the shRNA (Figure 5B). Significantly, while reintroduction of WT RNF8 restored 53BP1 focus formation in IR-treated cells (Figure 5C, left), and accumulation of conjugated ubiquitin in laser-generated DSB tracks (Figure 5C, right), both the *FHA (active but unable to accumulate at DSBs) and *RING (able to accumulate at DSBs but inactive) mutants failed to do so (Figure 5C). Thus, both MDC1-mediated accumulation of RNF8 and its RING-finger-associated ubiquitin ligase activity are required for local increase of DSB-associated ubiquitylations and the ensuing accumulation of repair proteins in this compartment.

DSB-Associated RNF8 Promotes Ubiquitylation of H2A

To elucidate the identity of the RNF8-mediated ubiquitin conjugates at DSBs, we applied fluorescence recovery after photobleaching (FRAP) (Lukas et al., 2004a; Bekker-Jensen et al., 2005) and analyzed the intranuclear dynamics of the WT and the catalytically-inactive (*RING) versions of RNF8. While the mobility of both proteins in the undamaged nucleoplasm was similar, their dynamics at the microlaser-generated DSBs were markedly different (Figure 6A). The mean residence time at DSBs of the inactive RNF8 extended up to 14.7 ± 0.1 s, which was more than three times longer than the WT protein (4.3 ± 0.1 s). Together with the biochemical analysis showing significant enrichment of the RING-deficient RNF8 in chromatin fractions (Figure S9A), these data suggest that the RNF8 substrate (from which the catalytically inactive

RNF8 may not readily dissociate) could be a constitutive component of chromatin.

To test this hypothesis, we examined whether RNF8 functionally interacts with H2A, a core histone that undergoes increased monoubiquitylation in response to UV-induced DNA damage (Bergink et al., 2006). Indeed, at least five independent pieces of evidence indicated a functional link between RNF8 and H2A. First, RNF8 physically interacted with H2A, and this interaction was much stronger after disrupting the RING domain of RNF8 (Figure 6B). Second, using an optimized assay to monitor histone ubiquitylation (Wang et al., 2006) we could detect an increase in the FLAG-H2A ubiquitylation of cells exposed to IR, and this increase was reduced after knocking down endogenous RNF8 (Figure 6C). Third, consistent with the requirement of MDC1 for RNF8 recruitment, MDC1 knockdown abrogated the IR-induced ubiquitylation of FLAG-H2A (Figure S9B). Fourth, the recombinant RNF8, but not the RING-deficient mutant, catalyzed ubiquitylation of H2A in an *in vitro* ubiquitylation assay (Figure 6D). Finally, an antibody specific to ubiquitylated H2A detected a clear accumulation of ubiquitin species at the microlaser-generated DSB sites, and this H2A-ubiquitin accumulation was inhibited by RNF8 knockdown (Figure 6E). Thus, H2A emerged as a strong candidate substrate of the DSB-associated RNF8 ubiquitin ligase. Interestingly, we noticed that depletion of RNF8 partially decreased also the overall H2A ubiquitylation even in nonirradiated cells (Figure 6C). Although we cannot exclude a damage-unrelated function of RNF8, this observation may also reflect spontaneous DNA breakage associated with replication errors.

To test whether RNF8 targets other histones, we extended the *in vitro* ubiquitylation assay and tested the ability of RNF8 to catalyze ubiquitylation of H2A, H2AX, H2B, and H3. While H2A and its structurally related variant H2AX were robustly ubiquitylated under these conditions, H2B and H3 were modified much less efficiently (Figure S9C). Interestingly, the ubiquitylation pattern of H2AX resembled that of H2A, and in both cases, WT RNF8 (but not the RING-deficient mutant) catalyzed attachment of multiple ubiquitin moieties (Figures 6D and S9C). Consistently, the oligo-ubiquitylation of FLAG-tagged H2A (Figure 6C) and to a lesser extent H2AX (Figure S9D) was also observed in cells, and the main ubiquitin species were induced by IR in an RNF8-dependent manner. Finally, similar oligo-ubiquitylated (and IR-inducible) H2A species were also observed on endogenous H2A immunopurified from native cell extracts by an antibody to ubiquitylated H2A (Figure S9E). Together, these data suggest that RNF8 increases local ubiquitylation of H2A (and to some extent also H2AX), and that these histone modifications closely correlate with the transition of the DSB-flanking chromatin to a state permissive for BRCA1 and 53BP1 accumulation.

RNF8 Facilitates Survival after a DSB-Generating Insult

Finally, we tested the impact of RNF8 on DNA-damage signaling and cell survival in an isogenic cell line capable

of conditional RNF8 knockdown. Reduction of RNF8 levels had little effect on cell-cycle progression in undamaged cells, indicating that the effect of RNF8 on BRCA1 and 53BP1 was not simply a consequence of a cell-cycle arrest (Figure S7C). In addition, the IR-induced phosphorylation of four independent ATM/ATR targets (SMC1, p53, CHK2, and CHK1) appeared normal (Figure S7D). This is consistent with our previous findings (Figures 3C and 4A) that RNF8, and the dynamic nuclear ubiquitylations, neither affect accumulation of MDC1 and NBS1 at the DSB sites (both events are known to facilitate ATM signaling [Falck et al., 2005; Lou et al., 2006; You et al., 2005]) nor interfere with the DSB resection and formation of the ssDNA compartments (Figure S7B), the structures required for activation of the ATR-Chk1 pathway (Cortez et al., 2001; Jazayeri et al., 2006). In agreement with these results, we did not observe significant defects in the cell-cycle checkpoints after RNF8 knockdown (our unpublished observations). We then tested whether the same degree of RNF8 downregulation would have any effect on clonogenic survival after a DSB-generating insult. When challenged with moderate doses of IR (2 Gy) the transient knockdown of RNF8 significantly reduced the fraction of surviving cells (Figure 6F). Together with the lack of obvious defects in ATM/ATR-mediated phosphorylations (Figure S7D) and the strong impairment of 53BP1 and BRCA1 focus formation in the absence of RNF8 (Figure 4A), these data suggest that the increased radiation sensitivity of RNF8-deficient cells reflects the inability to retain BRCA1 and 53BP1 (and possibly other genome surveillance factors) in the vicinity of the chromosomal lesions.

DISCUSSION

Our results suggest that RNF8 functions at the crossroads between two main stages of the DSB-induced chromatin response (Figure 7). While the first stage is competent to support DSB signaling, it is not permissive to increase local concentration of repair factors such as 53BP1 and BRCA1. This is only achieved in the second step, and the transition into this “mature” stage is critically dependent on the RNF8 ubiquitin ligase. The key question posed by this model is how does RNF8 promote local accumulation of proteins as diverse as 53BP1 and BRCA1? Two major scenarios come to mind. The first (“direct”) scenario assumes that the RNF8-mediated histone ubiquitylation recruits the molecular machinery that tethers both proteins to the DSB-modified chromatin. According to the second (“indirect”) scenario, RNF8 promotes a higher-order chromatin restructuring to facilitate local exposure of constitutive chromatin marks that in turn increase local affinity for 53BP1 and BRCA1. Below we discuss the fact that both models are plausible and not necessarily mutually exclusive.

The direct scenario is supported by the four recent studies showing that the ubiquitin-binding protein RAP80 is required to target BRCA1 to DSBs (Kim et al., 2007;

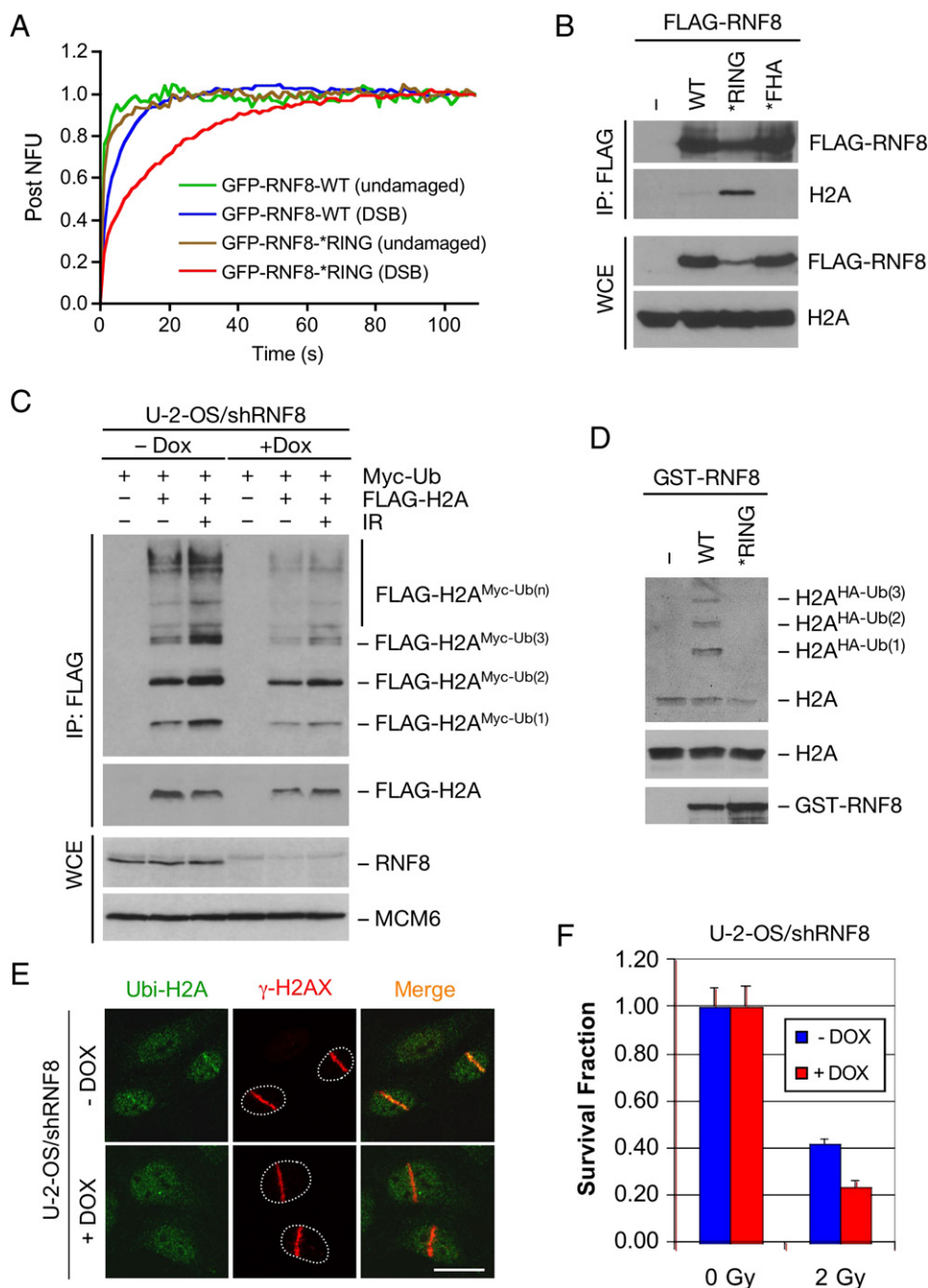


Figure 6. DSBs Trigger RNF8-Dependent Ubiquitylation of H2A

(A) U-2-OS cell lines stably expressing the indicated GFP-tagged RNF8 constructs were exposed to laser microirradiation. After 1 hr, the GFP-associated fluorescence was bleached in small rectangular regions ($2 \times 2 \mu\text{m}$) placed either to the undamaged nucleoplasm or over the laser-generated DSB tracks, and the fluorescence recovery in these regions was determined by repetitive image acquisition. The recovery curves were derived from 10 independent cells for each condition.

(B) HEK293T cells were transfected with indicated FLAG-RNF8 constructs. Cells were lysed in EBC buffer containing 500 mM NaCl, and extracts were subjected to immunoprecipitation with anti-FLAG agarose beads. Bound complexes were analyzed by immunoblotting with the indicated antibodies. (C) U-2-OS/shRNF8 cells were left untreated or induced by addition of Dox. Twenty-four hours later, cells were transfected with indicated constructs for an additional 24 hr and mock-treated or exposed to IR (20 Gy) for 1 hr. Total cell extracts were prepared by lysing cells in denaturing buffer and subjected to immunoprecipitation with anti-FLAG agarose beads under denaturing conditions. Bound complexes were analyzed by immunoblotting. (D) The capability of purified GST-RNF8 proteins to ubiquitylate Histone H2A in vitro was analyzed as described in [Experimental Procedures](#). Reaction mixtures were resolved by SDS-PAGE and analyzed by immunoblotting.

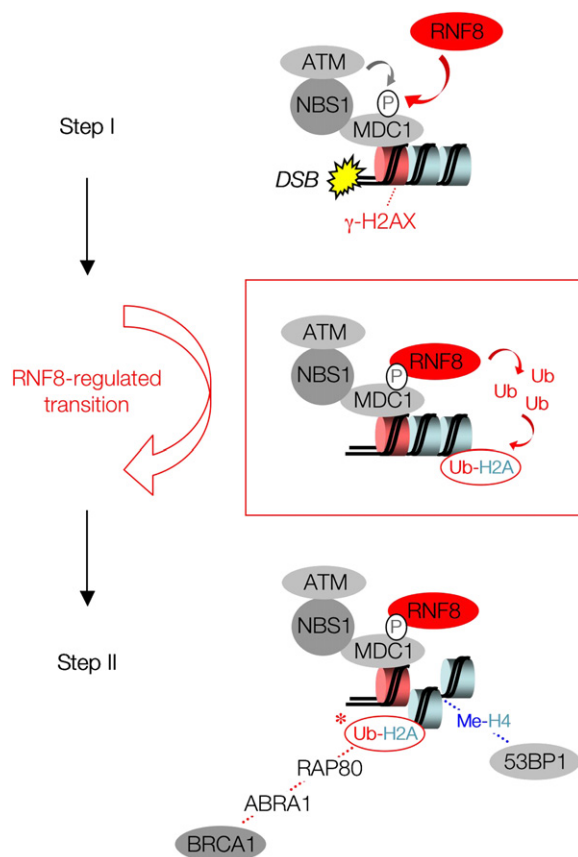


Figure 7. A Schematic Model of the Two-Step Chromatin Response to DSBs and the Involvement of RNF8 in This Process RNF8 recruitment to the DSB sites is triggered by the ATM-mediated phosphorylation of MDC1 (Step I). Once recruited, RNF8 catalyzes ubiquitylation of H2A. At this point, the DSB-flanking chromatin becomes competent to recruit and accumulate 53BP1 and BRCA1 (Step II). Asterisk indicates that H2AX, a structural variant of H2A, can also be targeted by RNF8 and may contribute to the transition between the initial and advanced stages of the DSB-induced chromatin response. See [Discussion](#) for details.

[Sobhian et al., 2007](#); [Wang et al., 2007](#); [Yan et al., 2007](#)). Although identification of the ubiquitin-dependent step in BRCA1 focus formation was important, none of these studies identified the enzymatic machinery that would locally ubiquitylate the DSB compartments and allow their recognition by RAP80. Our study may provide this missing link and allow us to describe the BRCA1 retention at the DSB-modified chromatin in a more complete fashion ([Figure 7](#)). Thus, the BRCA1 “assembly line” starts with phosphorylation of H2AX, followed by formation of the γ -H2AX-MDC1 complex, increased local concentration of ATM, phosphorylation of MDC1, and its recognition

by the FHA domain of RNF8. The resulting recruitment of RNF8 increases local ubiquitylation of histones such as H2A and H2AX, which may provide a direct recognition signal for the ubiquitin-interaction domains of RAP80. The entire pathway is then completed by ABRA1, a mediator protein that can simultaneously bind to RAP80 and the BRCT domains of BRCA1 ([Wang et al., 2007](#)).

In contrast to BRCA1, the mechanism of the DSB-induced accumulation of 53BP1 is less clear. While our results clearly implicate a ubiquitin-dependent step in this process, the link between the RNF8-mediated H2A/H2AX ubiquitylation and the Tudor-dependent binding of 53BP1 to methylated H3 or H4 remains elusive. One possibility is that RNF8, and the DSB-associated histone ubiquitylations, may also have an indirect impact on maturation of the DSB-flanking chromatin ([Figure 7](#)). In support for this indirect scenario, we noticed that genetic ablation of the TRRAP-TIP60 histone acetyltransferase (HAT) generated a phenotype that was remarkably similar to what we have observed after RNF8 knockdown ([Murr et al., 2006](#); this study). In each case, assembly of MDC1 at the DSB sites was normal but accumulation of both 53BP1 and BRCA1 was impaired. Because histone hyperacetylation is often coupled to the less compacted chromatin ([Kouzarides, 2007](#)), it is possible that local relaxation of the DSB-flanking regions contributes to the full-scale retention of BRCA1 and 53BP1 in this compartment, and that both histone ubiquitylation and acetylation contribute to this process. Our findings that RNF8 accumulates at DSBs extremely rapidly suggest that RNF8 may operate upstream of the TRRAP-TIP60 complex, possibly by stimulating its access to the DSB-flanking chromatin and/or by enhancing its HAT activity. Such an indirect scenario is attractive not only because it suggests a way to unmask cryptic methyl residues required for 53BP1 recruitment but also because it provides an opportunity to amplify the RAP80-mediated signal to locally concentrate BRCA1. It is important to realize that H2A and H2AX are also ubiquitylated in unstressed cells. However, it is also possible that in the context of intact interphase chromatin, the bulk of these ubiquitin conjugates may not be readily accessible to interact with ubiquitin-binding factors. We can imagine that even a modest increase of RNF8-mediated histone ubiquitylation (perhaps in conjunction with locally enhanced HAT activity) may alter chromatin configuration to an extent that would also expose the pre-existing histone-associated ubiquityl groups and thereby nucleate a more efficient “affinity trap” for RAP80 and indeed the entire machinery that concentrates BRCA1 in the vicinity of unrepaired DNA breaks.

A recent study reported H2A ubiquitylation after ultraviolet (UV)-induced DNA damage ([Bergink et al., 2006](#)).

(E) U-2-OS/shRNF8 cells were induced or not with Dox for 48 hr, exposed to laser microirradiation, and 1 hr later coimmunostained with antibodies to ubiquitylated H2A and γ -H2AX.

(F) U-2-OS/shRNF8 cells were induced or not with Dox for 24 hr, split by a limited dilution (+ Dox), and after an additional 24 hr exposed to the indicated doses of IR. Subsequently, the cells were incubated for an additional 14 days and stained with crystal violet. Colonies containing more than 50 cells were counted. The experiment was carried out in triplicates. Error bars represent standard error; scale bars, 10 μ m.

Although the UV-induced H2A ubiquitylation required ATR (conceptually similar to the ATM-dependent RNF8 regulation described here), some aspects of its regulation differed from the DSB response. Thus, the UV-induced H2A ubiquitylation was mediated by RNF2 (also known as RING1B), a canonical ubiquitin ligase required for H2A ubiquitylation associated with transcriptional silencing (de Napoles et al., 2004; Wang et al., 2004). In addition, it occurred relatively late after UV exposure and did not require H2AX (Bergink et al., 2006). Despite these differences (likely reflecting the fundamentally distinct repair machineries involved in DSBs and UV lesions), H2A ubiquitylation emerges as a general histone modification induced by DNA damage. While the rapid H2A ubiquitylation after DSBs serves to retain proteins engaged with various aspects of DSB repair, the delayed H2A ubiquitylation after UV may facilitate events that have high demands on a permissive chromatin configuration and are specific for UV-generated lesions such as repair of actively transcribed DNA strands.

Our findings, and the accompanying paper by Chen and colleagues, who reached similar conclusions (Huen et al., 2007 [this issue of *Cell*]), complement the emerging role of regulatory ubiquitylations in diverse aspects of the DNA-damage response by identifying an RNF8-dependent step during the maturation of the DSB-induced chromatin. We propose that the key purpose of the RNF8-controlled transition to the advanced stages of the DSB-induced chromatin response is to concentrate repair factors in the vicinity of DNA breaks to the threshold required for timely and accurate restoration of genome integrity. Both BRCA1 and 53BP1 are established tumor suppressors (Boulton, 2006; Morales et al., 2006; Ward et al., 2005). Identification of RNF8 as an upstream regulator of BRCA1 opens up the possibility that RNF8 might be targeted by mutations in a subset of breast and ovarian cancers, where the sequence of BRCA1 remains unaffected.

EXPERIMENTAL PROCEDURES

Plasmids and RNA Interference

A cDNA for human RNF8 (a gift from Yukio Okano) was inserted by PCR into pEGFP-C1 (Clontech) and pFLAG-CMV2 (Sigma), respectively. The *FHA (R42A) and *RING (C403S) mutations were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). An expression plasmid for HA-MDC1 (1–1100) was generated by insertion of a corresponding MDC1 PCR fragment into pcDNA4/TO (Invitrogen), containing two N-terminal HA-tags. The FLAG-H2A plasmid was a gift from Moshe Oren. Plasmid transfections were performed using FuGene 6 (Roche). siRNA oligonucleotides (Dharmacon) were synthesized to the following sequences (sense strand): RNF8-1 (5'-GGACAA UUAUGGACAACAA-3'), RNF8-2 (5'-UGCGGAGUAUGAAUAGAA-3'), MDC1 (Goldberg et al., 2003), and GL2 (control) (Mailand et al., 2006). All siRNA transfections were performed with 100 nM siRNA duplexes using Lipofectamine RNAiMAX (Invitrogen). A vector for inducible expression of RNF8-specific shRNA was generated by insertion of 5'-phosphorylated oligos containing the RNF8 target sequence (5'-AC ATGAAGCCGTTATGAAT-3') into pSUPERIOR.puro (Oligoengine). To generate shRNA-insensitive FLAG-RNF8 constructs, the underlined silent mutations (ACATGAGCGCGTTATGAAT) were introduced into the shRNF8 target sequence in the RNF8 coding region.

Cell Culture

Human U-2-OS osteosarcoma cells and 293T human embryonic kidney cells were grown in DMEM containing 10% fetal bovine serum (GIBCO). Where indicated, the culture medium was supplied by the proteasome inhibitor MG132 (Calbiochem; 5 μ M). U-2-OS cell lines stably expressing GFP-RNF8, FLAG-RNF8, or shRNF8 constructs in a Dox-inducible fashion were generated as described (Mailand et al., 2006). The U-2-OS cell lines stably expressing MDC1-shRNA, GFP-MDC1, GFP-53BP1, GFP-ATR, and NBS1-RFP were described (Bekker-Jensen et al., 2006; Jazayeri et al., 2006). U-2-OS cells stably expressing GFP-BRCA1 together with its heterodimerizing partner FLAG-BARD1 were generated using standard procedures (Bekker-Jensen et al., 2005; Lukas et al., 2004a) and are characterized in Figure S1. IR was delivered by the X-Ray generator (Pantak HF160, 150 kV, 15 mA, dose rate 2.18 Gy/min). Clonogenic survival assay is specified in the figure legends (Figure 7F).

Immunocytochemical Methods

Immunocytochemical assays were described (Bekker-Jensen et al., 2005, 2006; Lukas et al., 2004a; Mailand et al., 2006). Rabbit antiserum to RNF8 was raised against a peptide spanning residues 203–217 of human RNF8. Other antibodies included mouse and rabbit antibodies to HA-tag (sc-7392 and sc-805), rabbit polyclonal antibodies to GFP (sc-8334, Santa Cruz), histone H2A (Ab-18255, Abcam), RAP80 (BL2839, Bethyl Laboratories), phospho-SQ/TQ (2851, Cell Signaling), and mouse monoclonal antibodies to conjugated ubiquitin (FK2; Biomol, PW 8810) and to ubiquitylated H2A (05-678, Upstate). The specificity of the latter antibody for ubiquitylated H2A was demonstrated earlier (Baarends et al., 2005). Antibodies to FLAG, MCM6, γ -H2AX, Cyclin B1, MDC1, NBS1, 53BP1, and BRCA1 were described (Bekker-Jensen et al., 2006; Mailand et al., 2006).

Microscopy and Laser Microirradiation

Confocal images were acquired on LSM-510 (Carl Zeiss Microimaging Inc.) mounted on Zeiss-Axiovert 100M equipped with Plan-Neofluar 40 \times /1.3 oil immersion objective. Laser microirradiation to generate local DSBs and conditions for timelapse microscopy were described (Bekker-Jensen et al., 2005, 2006; Lukas et al., 2003, 2004a). FRAP assays including the mathematical analysis of the data were described previously (Lukas et al., 2004a).

Histone Ubiquitylation Assays

Histone ubiquitylation assays were performed essentially as described (Wang et al., 2006). Cells were cotransfected with FLAG-tagged histones and Myc-tagged ubiquitin and dissolved by sonication in denaturing buffer (20 mM Tris, pH 7.5; 50 mM NaCl, 0.5% NP-40; 0.5% Deoxycholate; 0.5% SDS; 1 mM EDTA) containing protease inhibitors. Cell extracts were subjected to immunoprecipitation with anti-FLAG M2 agarose beads (Sigma) under denaturing conditions and purified. Bound material was analyzed by immunoblotting. For *in vitro* ubiquitylation assays, 1 μ g of histones H2A, H2B, H3 (New England Biolabs), or H2AX (Upstate) was incubated in 30 μ l reaction mixture containing 50 mM Tris, pH 7.5; 5 mM MgCl₂; 2 mM NaF; 2 mM ATP; 10 μ M Okadaic acid; 1 mM DTT; 0.1 μ g E1; 0.2 μ g UbcH5c; and 1 μ g HA-ubiquitin (all from Boston Biochem). Two hundred nanograms of bacterially purified, full-length GST-RNF8 was added, and reactions were incubated for 1 hr at 37°C, stopped by the addition of Laemmli Sample Buffer, and resolved by SDS-PAGE. Ubiquitylated histones were detected by immunoblotting as specified in the figure legends.

Supplemental Data

Supplemental Data include nine figures and can be found with this article online at <http://www.cell.com/cgi/content/full/131/5/887/DC1/>.

ACKNOWLEDGMENTS

We thank Richard Baer, Junjie Chen, Thanos Halazonetis, Yukio Okano, and Moshe Oren for reagents and Junjie Chen for communicating unpublished results. This work was supported by the Danish Cancer Society, Danish National Research Foundation, European Union (integrated project "DNA Repair" and "Mutant p53"), European Science Foundation (EuroDYNA), Danish Research Council, SSMF Stockholm, and John and Birthe Meyer Foundation.

Received: March 12, 2007

Revised: June 11, 2007

Accepted: September 25, 2007

Published online: November 15, 2007

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Role of RNF8 in promoting assembly of DNA repair proteins

RNF8

RNF8 was previously described as a nuclear RING-finger protein (Ito et al., 2001), and an interaction partner and a transcription stimulator for the nuclear Retinoid X Receptor α (RXR α) (Takano et al., 2004). RNF8 contains a C-terminal RING-finger which has been reported to interact with different E2 enzymes to stimulate autoubiquitylation through K48 and K63 of ubiquitin (Ito et al., 2001; Plans et al., 2006). In addition, RNF8 contains an N-terminal phosphothreonyl-binding FHA domain, which is also found in Nbs1 and Mdc1.

In this study, RNF8 was found to accumulate in IR induced foci (IRIF) and in microlaser-generated DSB tracks at the DSB-flanking chromatin throughout interphase. The accumulation of RNF8 co-localized with and was dependent on γ -H2AX and Mdc1. In fact, RNF8 was found to be recruited to the site of damage through binding of its FHA domain to ATM/ATR mediated phosphorylated Mdc1. The accumulation of RNF8 to the site of damage was fast and with the same kinetics as Mdc1 and Nbs1. In comparison, BRCA1 and 53BP1 accumulated with slower kinetics, suggesting that RNF8 functions upstream of 53BP1 and BRCA1.

Treatment of cells with the proteasome inhibitor MG132 leads to a disruption of nuclear ubiquitin conjugates due to an accumulation of un-degraded polyubiquitylated proteins in the cytoplasm (Dantuma et al., 2006). In this study, immunostaining with an antibody, FK2, recognizing conjugated ubiquitin (mono and poly), revealed an accumulation of ubiquitin conjugates at laser-generated DSB tracks, which was lost upon treatment with MG132. Accumulation of BRCA1 and 53BP1 at the DSB-flanking chromatin was also found to be lost upon the treatment, suggesting a role of ubiquitylation in their recruitment. In contrast, γ -H2AX decoration of chromatin and recruitment of Mdc1, NBS1 and RNF8 to site of damage were un-affected. RNF8 depletion furthermore prevented recruitment of BRCA1 and 53BP1 and loss of DSB-associated ubiquitylations. RNF8 was therefore suggested to contribute to the DSB-induced ubiquitylations, which were required for the recruitment of BRCA1 and 53BP1 to the DSB-flanking chromatin.

RNF8 substrates

Fluorescence recovery after photo bleaching analysis (FRAP) suggested that the substrate of RNF8 was a constitutive component of chromatin, and indeed histones was found to be the target. Several *in vivo* and *in vitro* experiments pointed towards histone H2A as the main target together with histone H2AX, which was also suggested by (Huen et al., 2007). The histones were modified with several ubiquitin suggesting regulatory (poly)-ubiquitylation. Histones are monoubiquitylated in unstressed cells and RNF8 is suggested to promote additional ubiquitylations to alter the chromatin configuration to facilitate recruitment of 53BP1 and BRCA1. It would therefore be very interesting to investigate the ubiquitin modifications in more details.

RNF8 and Ubc13

A strong candidate for an E2 ubiquitin conjugating enzyme acting together with RNF8 in ubiquitylating H2A and H2AX is Ubc13. The RING-finger of RNF8 has earlier been shown to interact with Ubc13 and autoubiquitylation of RNF8 with K63 linkages requires Ubc13, suggesting that the two proteins act together (Plans et al., 2006). Originally, the heterodimer Ubc13/Mms2, acting together with E3 Rad5, was found to be involved in K63 polyubiquitylation of PCNA in post-replication repair in budding yeast (Hoege et al., 2002). Later it was observed that Ubc13 deficient human cells and chicken knockout cells exhibit chromosomal instability and hypersensitivity to both UV and IR, suggesting involvement of Ubc13 in post-replication repair of higher eukaryotes as well.

Ubc13 deficient cells were furthermore observed to be defective for HR-dependent repair, the recruitment of BRCA1 to DSBs was abolished, and the generation of RPA coated single stranded DNA was severely attenuated. It was therefore suggested that Ubc13 is involved in processes upstream of BRCA1, and that the recruitment and activity of BRCA1 requires Ubc13 dependent DSB processing (Zhao et al., 2007). If Ubc13 acts together with RNF8, its involvement in BRCA1 recruitment could be explained. Down-regulation of Ubc13 with siRNA also abolished IR induced foci formation of Rap80, Abraxas, 53BP1 and ubiquitin, and the same effect was observed when down-regulating RNF8 (Wang and Elledge, 2007). In addition, Ubc13 is so far the only E2 found to catalyze K63 ubiquitin chains.

Involvement of several ubiquitin ligases in ubiquitylation of the same substrate

Ring1B is the main protein of the polycomb group complex known to monoubiquitylate histone H2A under normal conditions (Buchwald et al., 2006), while RNF8 seems to act in response to DNA damage, and is recruited upon binding of Mdc1 to γ -H2AX. In our lab, an additional ubiquitin ligase RNF168 and another protein HERC2 were found to be involved in the ubiquitylation of histones upon DNA damage (Doil et al., in press; unpublished data). It could therefore be very interesting to investigate the interplay between RNF8, RNF168 and HERC2 and their regulation in more details. In the case of K63 polyubiquitylated PCNA in mammalian cells, monoubiquitylation of PCNA by Rad6/Rad18 is required for the second ubiquitin ligase, SHPRH, a human ortholog of yeast Rad5, to extend it to K63 polyubiquitylated PCNA, at least *in vitro* and in a mechanism dependent on Mms2-Ubc13 (Unk et al., 2006; Motegi et al., 2006). In addition, the HLTF ubiquitin ligase has been suggested to be a functional homolog of SHPRH and is also able to polyubiquitylate PCNA through K64 in the same manner as SHPRH (Motegi et al., 2008). It is therefore very likely that more factors are required for RNF8 to execute a full ubiquitin response.

Recruitment of BRCA1

BRCA1 is found in three different complexes with Abraxas, BACH1 or CtIP which bind to the BRCT domains of BRCA1 in their phosphorylated state, and in a mutually exclusive manner. The exact role of BRCA1 in the different complexes is still not entirely clear. Rap80 is involved in recruiting the Abraxas complex to the site of damage (Wang et al., 2007). Since Rap80 is suggested to bind K63 ubiquitin chains through its UIM domains (Kim et al., 2007a; Sobhian et al., 2007), an interesting model is that Rap80 binds to K63 ubiquitin chains of histones to recruit Abraxas, BRCA1/BARD1 and BRCC36 to the site of damage, which is also suggested by Wang and Elledge, 2007. But the proof of a direct interaction between Rap80 and ubiquitin modifications of histones, processed by RNF8, is still missing. It is also very interesting that beside RNF8, BRCC36 is required for BRCA1 recruitment to chromatin and the K63 DUB activity of BRCC36 could suggest that “editing” of local ubiquitin is necessary (Chen et al., 2006).

Recruitment of 53BP1

The recruitment of 53BP1 to site of damage is less clear than the BRCA1. 53BP1 has no identified ubiquitin binding domains and currently, it is proposed that 53BP1 binds dimethylated K20 of histone H4 or dimethylated K79 of histone H3, directly through its Tudor domain in a manner dependent on DNA damage (Botuyan et al., 2006; Huyen et al., 2004). The data from our study clearly indicates the involvement of an ubiquitin dependent step in the recruitment, which requires RNF8. Based on this, RNF8 is suggested to promote changes in the chromatin by its ubiquitylation activity to provide a less compact chromatin, allowing 53BP1 access to methylated H4 or H3. But the exact mechanism of the change in chromatin topology facilitated by H2A ubiquitylation needs to be analyzed in more details. Other factors are likely to be involved including the suggested TRRAP-TIP60 histone acetyltransferase which bind DSB-flanking chromatin, and is suggested to contribute to a less compact chromatin by acetylation of histones to facilitate access and binding of 53BP1 and

BRCA1 to site of damage (Murr et al., 2006). Furthermore, other proteins could contribute to bridge the RNF8 mediated ubiquitylation with the 53BP1 recruitment.

Two different mechanisms of recruitment are therefore likely to apply for BRCA1 and 53BP1. BRCA1 appears to be directly recruited to RNF8 mediated ubiquitylations, whereas 53BP1 is indirectly recruited. The effect of RNF8 depletion on survival, but not the checkpoints suggest a repair defect and a role of 53BP1 and BRCA1 in DSB repair. It is therefore interesting that BRCA1 and 53BP1 interact with the chromatin flanking region, although BRCA1 also interact with the single stranded DNA micro-compartment. This could perhaps contribute to a better understanding of their exact functions.

With the presented data, RNF8 is emerging as an important factor necessary for modification of DNA damage flanking chromatin compartment, and to facilitate recruitment of BRCA1 and 53BP1 to the site of damage. Furthermore, the exact mechanism of ubiquitylation of histones seems to be more complex than previous anticipated. It is very likely that more factors will emerge in the future and shed more light on the mechanism of protein retention at DNA damage sites. Furthermore this could help us to understand the complex network of the DNA damage response applied by cells to maintain genomic stability.

4.2 Publication 2

Helene Faustrup, Simon Bekker-Jensen, Jiri Bartek, Jiri Lukas and Niels Mailand. USP7 counteracts SCF ^{β TrCP} - but not APC^{Cdh1} -mediated proteolysis of Claspin. *Journal of Cell Biology* 184, 13-19, 2009.

USP7 counteracts SCF^{βTrCP}- but not APC^{Cdh1}-mediated proteolysis of Claspin

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Claspin is an adaptor protein that facilitates the ataxia telangiectasia and Rad3-related (ATR)-mediated phosphorylation and activation of Chk1, a key effector kinase in the DNA damage response. Efficient termination of Chk1 signaling in mitosis and during checkpoint recovery requires SCF^{βTrCP}-dependent destruction of Claspin. Here, we identify the deubiquitylating enzyme ubiquitin-specific protease 7 (USP7) as a novel regulator of Claspin stability. Claspin and USP7 interact in vivo, and USP7 is required to maintain steady-state levels of Claspin. Furthermore, USP7-mediated deubiquitylation markedly prolongs the

half-life of Claspin, which in turn increases the magnitude and duration of Chk1 phosphorylation in response to genotoxic stress. Finally, we find that in addition to the M phase-specific, SCF^{βTrCP}-mediated degradation, Claspin is destabilized by the anaphase-promoting complex (APC) and thus remains unstable in G1. Importantly, we demonstrate that USP7 specifically opposes the SCF^{βTrCP}- but not APC^{Cdh1}-mediated degradation of Claspin. Thus, Claspin turnover is controlled by multiple ubiquitylation and deubiquitylation activities, which together provide a flexible means to regulate the ATR-Chk1 pathway.

Introduction

In response to genotoxic stress, eukaryotic cells elicit DNA damage checkpoint responses, which delay cell cycle progression and stimulate DNA repair to restore genomic integrity (Zhou and Elledge, 2000; Bartek and Lukas, 2007). DNA damage triggers rapid degradation of the Cdk-activating phosphatase, Cdc25A, by the SCF^{βTrCP} ubiquitin ligase to arrest the cell cycle in a reversible fashion (Mailand et al., 2000; Busino et al., 2003). This process requires priming phosphorylation of Cdc25A by the checkpoint kinase Chk1, which is itself activated by phosphorylation by the upstream kinase ataxia telangiectasia and Rad3-related (ATR). Efficient ATR-mediated phosphorylation of Chk1 occurs only in the presence of the checkpoint mediator Claspin, a key determinant for Chk1 activation (Kumagai and Dunphy, 2000). Upon entry into mitosis and during recovery from DNA damage-induced cell cycle arrest, Claspin undergoes proteasomal degradation, and such control of Claspin levels plays a pivotal role in restraining Chk1 activity under these conditions (Mailand et al., 2006; Mamely et al., 2006; Peschiaroli et al., 2006). Like in the case of Cdc25A, the destruction of Claspin is also mediated by SCF^{βTrCP}; hence, this

complex plays a key role in initiating as well as terminating DNA damage checkpoints. These findings have helped to establish regulated ubiquitylation as a major signaling mechanism in the DNA damage response.

The removal of ubiquitin conjugates from target proteins by deubiquitylating enzymes (DUBs) has emerged as an important regulatory mechanism in a range of cellular processes. An estimated 79 functional DUBs are encoded by the human genome, but as yet, only few of these have been assigned functions or substrates (Nijman et al., 2005b). Available evidence suggests that the specificity and regulatory potential of DUBs may be comparable to that of E3 ubiquitin ligases, underscoring the dynamic and reversible nature of protein ubiquitylation. Several DUBs have been found to function in the DNA damage response, including ubiquitin-specific protease 1 (USP1), USP7, and USP28 (Nijman et al., 2005a; Huang et al., 2006; Zhang et al., 2006). For instance, USP7 (also known as HAUSP) is a DUB for Mdm2 (Li et al., 2004), a ubiquitin ligase for p53, and is thus an important factor in the control of p53 abundance during the cell cycle.

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Abbreviations used in this paper: APC, anaphase-promoting complex; ATR, ataxia telangiectasia and Rad3-related; CI, catalytically inactive; DOX, doxycycline; DUB, deubiquitylating enzyme; HU, hydroxyurea; IB, immunoblotting; IP, immunoprecipitation; USP, ubiquitin-specific protease; WT, wild type.

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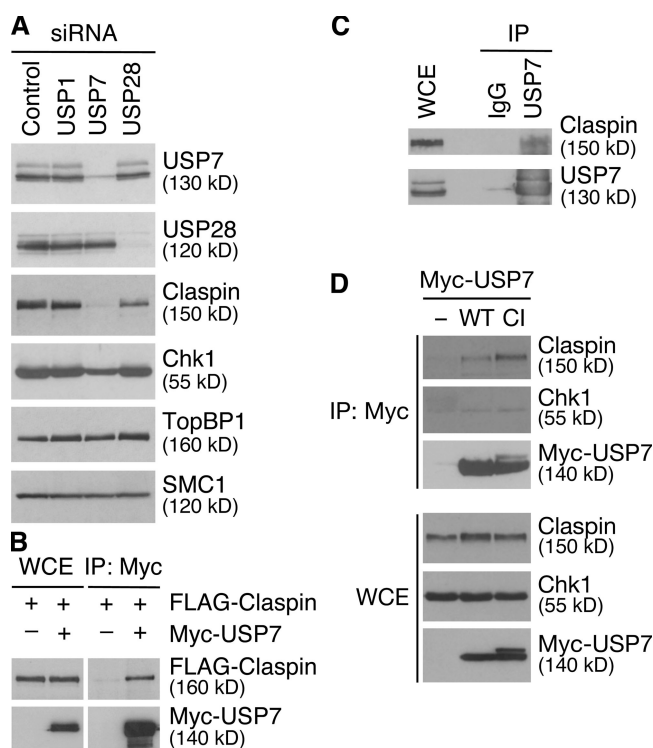


Figure 1. Claspins interact with USP7. (A) U2OS cells were transfected with indicated siRNAs for 72 h and analyzed by IB with the indicated antibodies. The functionality of the USP1 siRNA has been demonstrated previously (Huang et al., 2006). (B) Lysates of 293T cells transfected with indicated plasmids for 24 h were subjected to Myc IP followed by IB. (C) Lysates of 293T cells were subjected to IP with USP7 antibody or preimmune rabbit serum (IgG) followed by IB. (D) 293T cells were transfected with WT or CI forms of Myc-USP7, and processed as in B. The extra band in the USP7 CI lane corresponds to ubiquitinated USP7 (unpublished data).

In this report, we show that the control of Claspins stability is more complex than previously anticipated, involving both ubiquitylation and deubiquitylation to allow cells to finely gauge the levels of Claspins. We identify USP7 as a major DUB for Claspins, with a consequent impact on Chk1 phosphorylation and checkpoint recovery. We also demonstrate that Claspins are degraded by the anaphase-promoting complex (APC) in the G1 phase, and that USP7 specifically counteracts SCF^{βTRCP}- but not APC^{Cdh1}-mediated ubiquitylation of Claspins.

Results and discussion

Claspins interact with USP7

To explore the dynamic control of Claspins stability, we investigated whether Claspins are regulated by deubiquitylation activities. Focusing on a selected set of DUBs, which have previously been implicated in the DNA damage response, we monitored the response of Claspins levels to siRNA-mediated down-regulation of these DUBs. Consistent with published results (Zhang et al., 2006), we observed a partial reduction in Claspins level after knocking down USP28 (Fig. 1 A). Strikingly, however, we consistently detected a much more prominent decrease of Claspins expression after depletion of USP7, which suggests that USP7 might protect Claspins from ubiquitylation-dependent degradation (Fig. 1 A). However, the abundance of TopBP1, which is

also required for Chk1 activation in response to genotoxic insults, was not affected by knockdown of USP7 or other DUBs (Fig. 1 A). A mixture of three independent oligonucleotides was used to efficiently knock down USP7 expression, and the effect of USP7 depletion on Claspins levels could be reproduced with individual siRNAs to USP7 (Fig. S1 A, available at <http://www.jcb.org/cgi/content/full/jcb.200807137/DC1>). To clarify whether Claspins is a direct target of USP7, we tested whether the two proteins copurify in immunoprecipitation (IP) experiments. Indeed, USP7 and Claspins readily interacted under conditions where both proteins were overexpressed (Fig. 1 B), and we could also detect association between the endogenous proteins (Fig. 1 C). In contrast, we did not observe binding of Claspins to several other DUBs (unpublished data), underscoring the specificity of the Claspins-USP7 interaction. To further probe the relationship between USP7 and Claspins, we assessed the Claspins-binding capability of wild-type (WT) or catalytically inactive (CI) USP7. In such experiments, USP7 CI interacted more strongly with endogenous Claspins than did WT USP7 (Fig. 1 D), which is consistent with a substrate-trapping mechanism in which an inability of inactive USP7 to deubiquitylate Claspins would manifest as a prolonged binding and thus a tighter interaction. These observations suggest that Claspins is a novel substrate for USP7.

Interestingly, depletion of USP7 also led to a significant down-regulation of total Chk1 levels (Fig. 1 A), but we failed to produce credible evidence that Chk1 is a direct target for USP7. In particular, whereas Claspins avidly interacted with USP7, the amount of Chk1 coimmunoprecipitated with ectopic USP7 did not significantly exceed that observed in the control cells (Fig. 1 D). In addition, knockdown of Claspins or Chk1 negatively affected the expression level of the other protein (Fig. S1 C), which suggests that Claspins and Chk1 may promote the stability of each other, in agreement with previous findings (Yang et al., 2008). Thus, it seems likely that the effects on Chk1 levels observed in response to up- or down-regulation of USP7 may be indirectly mediated through its impact on Claspins.

USP7 deubiquitylates and stabilizes Claspins

To corroborate the emerging link between USP7 and Claspins, we generated cell lines capable of conditionally expressing WT or CI mutant forms of Myc-tagged USP7. Induction of USP7 in these cell lines resulted in homogenous nuclear expression of the transgenes in virtually all cells, but had little impact on cell cycle distribution (Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200807137/DC1>; and not depicted). Using these cell lines, we asked whether overexpression of USP7 would have a stabilizing effect on endogenous Claspins. Expression of USP7 WT clearly resulted in an elevation of Claspins levels, whereas no such effect could be seen in cells induced to express USP7 CI (Fig. 2 A). Only a slight increase in Chk1 levels was evident under these conditions, which further supports the notion that Claspins rather than Chk1 is a target of USP7. As a control for the functionality of ectopic USP7 in the cell lines, we analyzed its impact on Mdm2, a known target for USP7. Indeed, Mdm2 abundance was strongly elevated in a manner dependent on the catalytic activity of USP7 but independent of cell cycle

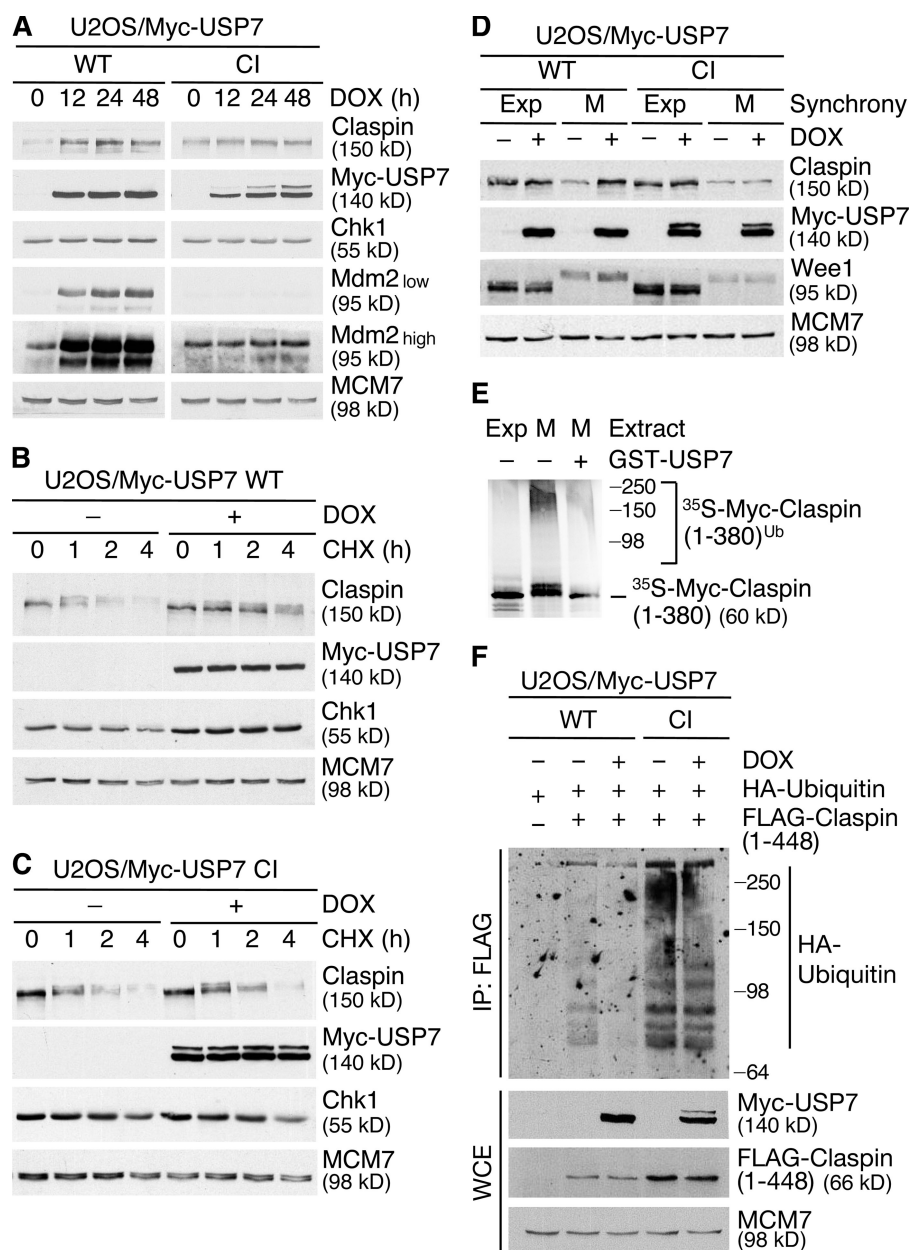


Figure 2. USP7 deubiquitylates and stabilizes Claspins. (A) U2OS/Myc-USP7 cell lines were induced with doxycycline (DOX), harvested at the indicated times, and processed for IB with the indicated antibodies. (B) U2OS/Myc-USP7 WT cells were induced or not induced with DOX for 30 h, after which cycloheximide (CHX) was added to the cultures for the indicated times. The half-life of Claspins was estimated by IB of total cell extracts. (C) U2OS/Myc-USP7 CI cells were treated as in B. (D) U2OS/Myc-USP7 WT or CI cells were induced or not induced for 18 h with DOX, and left untreated (Exp) or incubated with nocodazole for an additional 12 h to synchronize cells in mitosis (M). The cell extracts were then analyzed by IB. Mobility-shifted Wee1 served as a marker for mitotically synchronized cells. (E) [³⁵S]-labeled Claspins (amino acids 1–380) was incubated in ubiquitylation reaction mix, then supplemented with extracts of exponentially growing or mitotic U2OS cells and in vitro-translated β TrCP1. Where indicated, bacterially purified GST-USP7 WT was added to the reaction. Claspins ubiquitylation was visualized by autoradiography. Numbers to the right of the gel blots indicate molecular mass standards in kD. (F) U2OS/Myc-USP7 cell lines were transfected with indicated constructs for 24 h, and lysates were processed for IP with FLAG antibody and IB.

stage (Fig. 2 A and Fig. S2 C). To clarify further whether Claspins are stabilized by USP7-dependent deubiquitylation, we assessed the half-life of Claspins in cells expressing ectopic USP7 by adding cycloheximide to block protein synthesis. Overexpression of USP7 WT brought about a robust, three- to fourfold increase in the stability of Claspins as compared with uninduced cells, in which the half-life of Claspins was very short (~ 1 h, Fig. 2 B). In contrast, little if any effect on Claspins stability was evident in cells expressing USP7 CI (Fig. 2 C), which supports the idea that Claspins are deubiquitylated by USP7.

USP7 reverses SCF β TrCP-dependent ubiquitylation of Claspins

We have previously shown that the levels of Claspins oscillate in a cell cycle-dependent manner, being high in S and G2 phases and declining sharply upon entry into mitosis and throughout

G1 (Mailand et al., 2006). Degradation of Claspins at the onset of mitosis is driven by its ubiquitylation by SCF β TrCP, and we therefore asked whether overexpression of USP7 could counteract this process. To this end, we assessed the impact of ectopic USP7 on the levels of Claspins in mitotic cells. In uninduced cells, Claspins were indeed expressed at much lower levels in mitotic cells than in exponentially growing cells (Fig. 2 D). However, as would be expected if USP7 was able to reverse SCF β TrCP-mediated ubiquitylation of Claspins, induction of USP7 WT fully restored Claspins levels to those seen in asynchronous cells (Fig. 2 D). Again, this propensity of USP7 was dependent on the catalytic activity of USP7, as the abundance of Claspins remained low in mitotic cells expressing USP7 CI (Fig. 2 D). Because extracts from mitotic cells readily support ubiquitylation of Claspins in a β TrCP-dependent fashion (Mailand et al., 2006), we used such an approach to test whether USP7 directly

deubiquitylates Claspin in vitro. As shown in Fig. 2 E, the polyubiquitylation of Claspin observed in mitotic cells was inhibited when purified GST-tagged USP7 WT was added to the reaction. In addition, induction of USP7 WT but not USP CI quantitatively suppressed the appearance of polyubiquitylated Claspin species in cells (Fig. 2 F), which indicates that USP7 is a major Claspin-directed DUB. Together, these results demonstrate that USP7 stabilizes Claspin by directly opposing its β TrCP-mediated ubiquitylation. Hence, the balance of ubiquitylation and deubiquitylation activities allow for subtle control of steady-state levels of Claspin.

Claspin is degraded by the APC in G1

Although elevated levels of USP7 WT were able to maintain Claspin expression during mitosis, we noted that it was still degraded upon reentry into G1 phase (Fig. 3 A). This suggested that once in G1, Claspin becomes susceptible to degradation by a β TrCP-independent mechanism refractory to USP7 activity. We reasoned that under these conditions, the destruction of Claspin might instead be driven by the APC, which promotes the degradation of numerous regulatory proteins in late mitosis and G1 (Peters, 2006). To test this possibility, we coexpressed Claspin with Cdh1, the substrate-specific activator of the APC in G1, using the finding that elevated levels of Cdh1 are sufficient to trigger APC activation irrespective of cell cycle stage (Sorensen et al., 2000). In the presence of high levels of Cdh1, the expression of Claspin was strongly suppressed (Fig. 3 B), which suggests that Claspin is indeed a target for APC-mediated degradation. To further probe this proteolytic mechanism, we used an immunofluorescence-based approach to monitor Claspin abundance in G1 cells. Although Claspin was detectable only at background levels in G1 phase (Cyclin B1-negative) nuclei, treatment with a proteasome inhibitor (MG132) was sufficient to restore Claspin expression to levels comparable with S and G2 phase cells (Fig. 3 C), demonstrating that Claspin is actively degraded by the proteasome in G1. Next, we subjected cells to siRNA-mediated depletion of β TrCP or Cdh1 to test whether the degradation of Claspin in G1 cells was predominantly mediated by SCF $^{\beta$ TrCP or APC Cdh1 . Although Claspin did not accumulate in G1 cells in response to knockdown of β TrCP, its expression in G1 was efficiently restored upon depletion of Cdh1 (Fig. 3 C), indicating that the degradation of Claspin in G1 phase is mediated by APC Cdh1 but not SCF $^{\beta$ TrCP. In contrast, we had previously shown that the APC was unable to promote the degradation of Claspin in early mitosis (Mailand et al., 2006). Thus, two distinct pathways operate to limit Claspin abundance during the cell cycle, in a manner much like Cdc25A, whose destruction is also controlled by both SCF $^{\beta$ TrCP and APC Cdh1 (Donzelli et al., 2002). We speculate that the APC-mediated degradation of Claspin in G1 is an important means of suppressing inappropriate Chk1 activation during this window of the cell cycle. Indeed, cells expressing elevated levels of Cdh1 were markedly impaired in their ability to activate Chk1 in response to UV, whereas the phosphorylation of other ATR targets remained virtually normal, as exemplified by p53 (Fig. 3 D). The choice of APC as the machinery for Claspin ubiquitylation in G1 may reflect the

fact that priming phosphorylation of Claspin by the Plk1 kinase is required for its SCF $^{\beta$ TrCP-mediated destruction (Mailand et al., 2006; Mamely et al., 2006; Peschiaroli et al., 2006); the absence of Plk1 in G1, because of its destruction by APC Cdh1 (Peters, 2006), thus necessitates a Plk1-independent mechanism for the G1-specific degradation of Claspin.

As mentioned previously, the APC Cdh1 -mediated degradation of Claspin appeared insensitive to USP7 activity (Fig. 3 A), and to confirm this, we tested the ability of APC Cdh1 to degrade Claspin in the presence of elevated levels of USP7. Unlike inactivation of APC Cdh1 , expression of ectopic USP7 WT did not restore Claspin expression in G1 (Fig. 3 E), which indicates that the stabilization of Claspin observed in these cells (Fig. 2, A and B) happens outside G1 phase. In addition, overexpressed USP7 failed to counteract Claspin degradation mediated by activated APC Cdh1 (Fig. 3 F). We conclude from these experiments that USP7 selectively opposes the SCF $^{\beta$ TrCP- but not APC Cdh1 -dependent degradation of Claspin, which suggests that the molecular nature of ubiquitin chains attached to Claspin by APC Cdh1 and SCF $^{\beta$ TrCP differ in terms of their susceptibility or accessibility to USP7-mediated deubiquitylation.

USP7 controls the timing of checkpoint-induced Chk1 phosphorylation through regulation of Claspin stability

The role of USP7 in maintaining steady-state levels of Claspin suggested that modulation of USP7 activity might affect Chk1 regulation during checkpoint responses. To test this, we first assessed the ability of USP7-depleted cells to activate Chk1. Consistent with the quantitative loss of Claspin in such cells, knockdown of USP7 strongly impaired UV-dependent activation of Chk1, as judged from its phosphorylation on Ser317 (Fig. 4 A). As with Claspin levels, this effect was specific to USP7, as neither depletion of USP1 or USP28 significantly compromised UV-induced Chk1 phosphorylation (Fig. 4 A). Hence, these data suggest that USP7 is required for Chk1 activation in response to DNA damage.

Destruction of Claspin by SCF $^{\beta$ TrCP promotes the timely inactivation of Chk1 during recovery from DNA damage-induced cell cycle arrest (Mailand et al., 2006; Mamely et al., 2006; Peschiaroli et al., 2006), and because USP7 protects Claspin from β TrCP-dependent degradation, we reasoned that the activity of USP7 might be a contributing factor in timing the duration of Chk1 phosphorylation during checkpoint responses. To this end, we analyzed the kinetics of Chk1 dephosphorylation, and thus checkpoint termination, in USP7 cell lines released from a replication block induced by hydroxyurea (HU). We consistently observed a strong delay in the ability of USP7 WT-induced cells to degrade Claspin and inactivate Chk1 after release from the HU-induced arrest, relative to uninduced cells (Fig. 4 B). This was accompanied by a slower rate of cell cycle progression upon HU removal, which might at least partially reflect the delayed kinetics of Claspin degradation in these cells (Fig. S3, available at <http://www.jcb.org/cgi/content/full/jcb.200807137/DC1>). The overall levels of Chk1 remained somewhat higher in cells induced to express USP7 WT, which is in agreement with the previously observed correlation

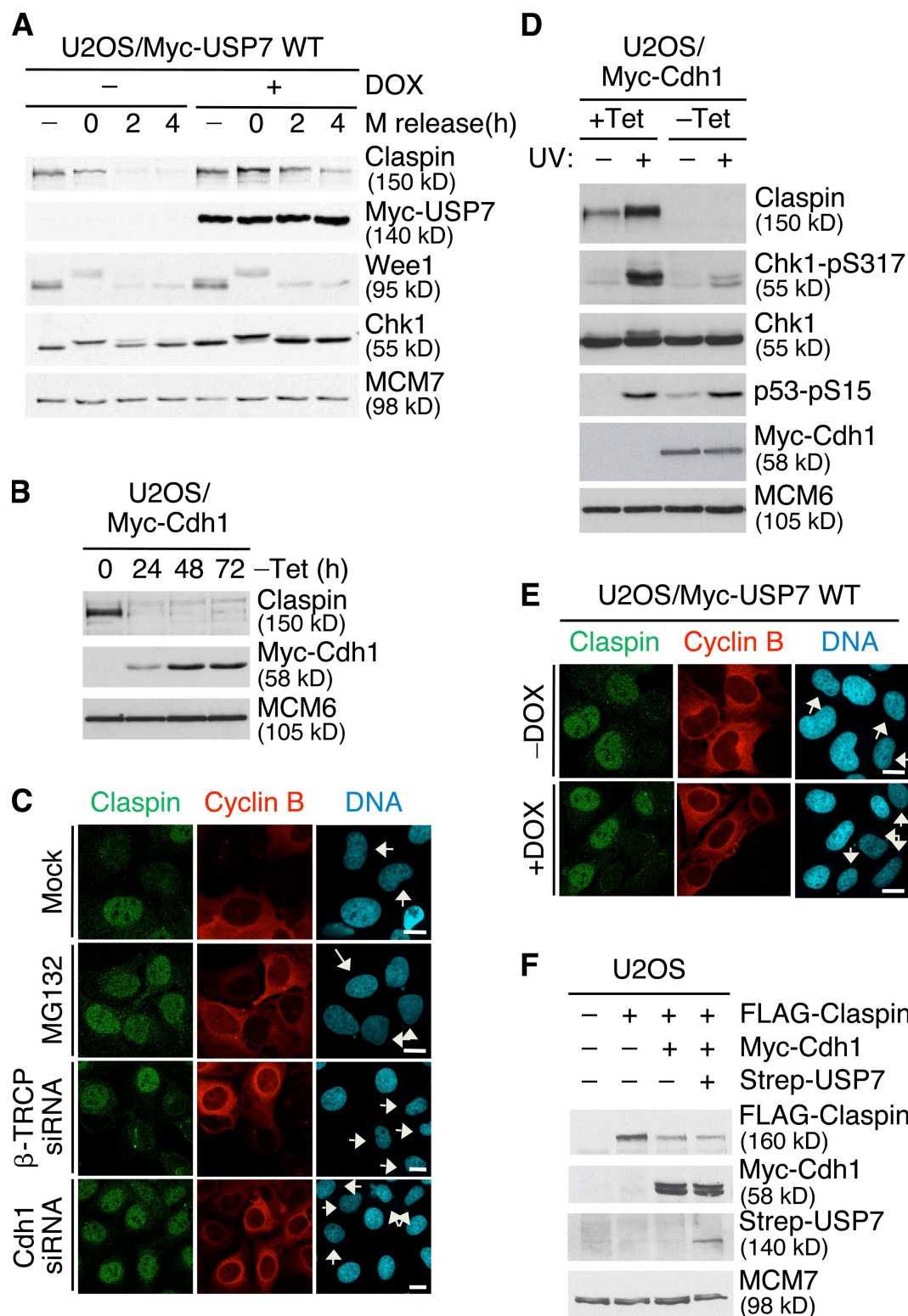
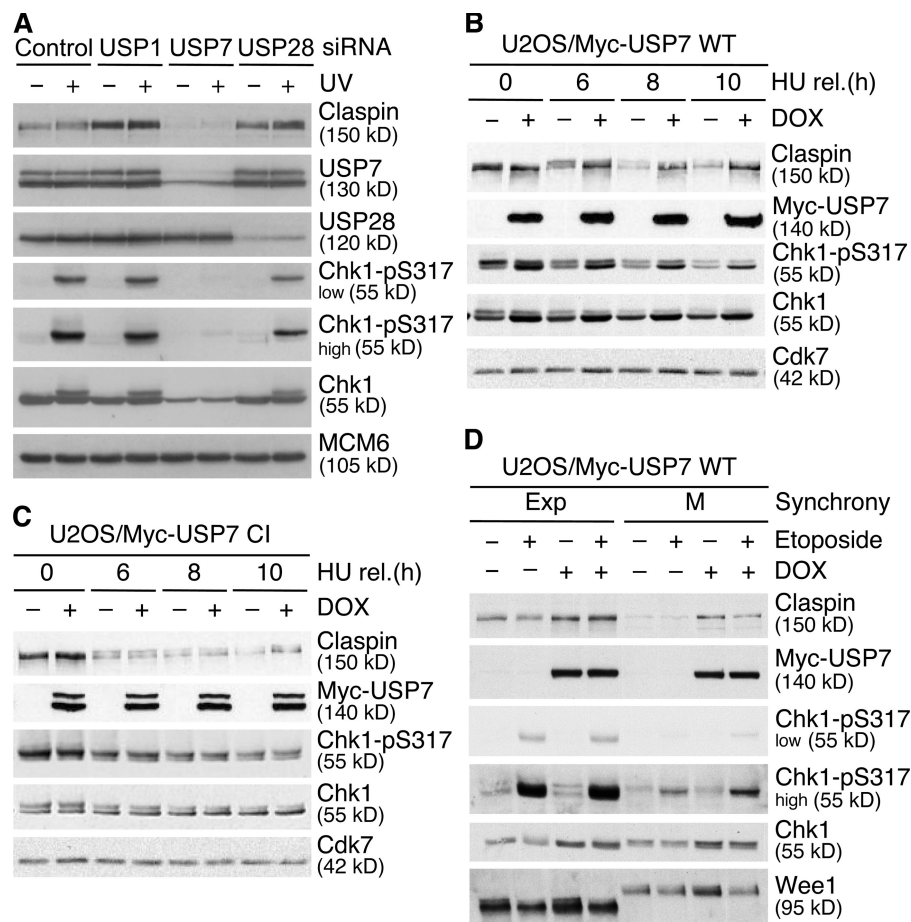


Figure 3. Degradation of Claspin by APC^{Cdh1} in G1 is not opposed by USP7. (A) U2OS/Myc-USP7 WT cells were induced or not induced with DOX for 18 h, and left untreated or synchronized in mitosis by nocodazole treatment for an additional 12 h. Mitotic cells were washed, plated in fresh medium, and collected at the indicated times after release. Lysates of these and asynchronous control cells (-) were analyzed by IB. (B) U2OS/Myc-Cdh1 cells were induced by removal of tetracycline (Tet) for the indicated times and processed for IB. (C) U2OS cells were treated with MG132 for 4 h or transfected with β TRCP or Cdh1 siRNAs for 48 h. The cells were then fixed and immunostained with indicated antibodies. DNA was visualized by counterstaining with ToPro3. Arrows indicate G1 cells. Bars, 10 μ m. (D) U2OS/Myc-Cdh1 cells were induced or not induced by tetracycline withdrawal for 48 h, exposed to UV (20 J/m²), and harvested 1 h later. Total cell extracts were processed for IB. (E) U2OS/Myc-USP7 WT cells were induced or not induced with DOX for 48 h, and processed for immunofluorescence as in C. Arrows indicate G1 cells. Bars, 10 μ m. (F) U2OS cells were transfected with the indicated plasmids for 24 h and processed for IB.

Figure 4. USP7 controls the timing of Chk1 phosphorylation through regulation of Claspin stability. (A) U2OS cells were transfected with indicated siRNAs for 72 h, then subsequently treated with UV (20 J/m²) and harvested 1 h later. Cells were then processed for IB. (B) U2OS/Myc-USP7 WT cells were induced or not induced with DOX for 12 h, and treated with HU for an additional 24 h. Cells were then released from the HU block, harvested at the indicated times, and processed for IB. (C) U2OS/Myc-USP7 CI cells were treated as in B. (D) U2OS/Myc-USP7 WT cells were induced or not induced with DOX for 24 h, and left untreated or synchronized in mitosis with nocodazole for an additional 12 h. Cells were then treated with etoposide for 2 h where indicated, and processed for IB.



between Chk1 and Claspin levels. Consistent with the inability of USP7 CI to stabilize Claspin, cells expressing this mutant did not display any checkpoint recovery delay (Fig. 4 C). Hence, increased activity of USP7 interferes with the cellular ability to timely degrade Claspin and inactivate Chk1 upon checkpoint termination.

In addition to its role during checkpoint recovery, β TrCP-dependent degradation of Claspin helps prevent inappropriate activation of Chk1 during mitosis (Mailand et al., 2006; Mamely et al., 2006). Consequently, mitotic cells depleted for β TrCP display partial Chk1 phosphorylation after exposure to genotoxic agents. Because USP7 stabilized Claspin in mitosis, we tested if mitotic cells overexpressing USP7 WT would allow Chk1 activation. Indeed, like β TrCP depletion, elevated levels of USP7 WT partially enabled phosphorylation of Chk1 upon exposure of mitotic cells to the DNA-damaging agent etoposide (Fig. 4 D). These observations suggest that the ubiquitylation and deubiquitylation activities toward Claspin must be tightly coordinated to enforce Claspin degradation and thus Chk1 inactivation in mitosis.

Collectively, our results uncover a dynamic and complex mode of controlling Claspin stability during the cell cycle and upon checkpoint-inducing stimuli involving both ubiquitylation and deubiquitylation activities. These findings highlight the tuning of Claspin availability as a key regulatory event in pathways that govern Chk1 activation, and likely reflect the fact that Claspin is specifically required for ATR-mediated phosphoryla-

tion of Chk1 but not other targets (Liu et al., 2006). Most importantly, we identified USP7 as a DUB opposing β TrCP-mediated ubiquitylation of Claspin, thus broadening the scope of USP7 functions in the maintenance of genomic integrity. By protecting Claspin from degradation, USP7 may directly impact the initiation as well as termination of Chk1-mediated signaling responses, and hence it will be important to address if and how the Claspin-directed activity of USP7 is itself regulated during checkpoint responses. A recent study also identified Claspin as a novel APC target, and the DUB USP28 was found to oppose its APC-mediated ubiquitylation (Bassermann et al., 2008). Hence, two distinct DUBs may be used to counteract Claspin ubiquitylation mediated by SCF $^{\beta$ TrCP or APC Cdh1 , further highlighting the importance of tightly controlling its steady-state expression levels during the cell cycle.

Materials and methods

Plasmids and RNA interference

Plasmids expressing WT and CI [C223S] Myc-tagged USP7 (pcDNA3-Myc-USP7) were gifts from R. Everett (MRC Virology Unit, University of Glasgow, Scotland, UK). The inserts were inserted into pcDNA4/TO (Invitrogen), allowing for doxycycline-inducible expression of Myc-USP7 WT and CI, and into pGEX-20T (GE Healthcare) for bacterial expression of GST-USP7 fusion proteins. Other plasmids used in this study included pRES-FLAG-Claspin, pCMV2-FLAG-Claspin (amino acids 1–448), pX-Myc-Claspin (amino acids 1–380), pX-Myc-Cdh1, and pX-Myc- β TrCP1, all of which have been described previously (Mailand et al., 2006; Sorensen et al., 2000). Plasmid transfections were performed using FuGene6 (Roche).

A mixture of three different siRNAs were used to efficiently knock down USP7, as described previously (Canning et al., 2004). Other siRNAs used in this study included USP1 (5'-GGCAAUACUUGCUAUCUUA-3'; Nijman et al., 2005a) and USP28 (5'-CUGCAUUCACCUUAUUAU-3'). siRNA to Cdh1 and β TrCP have been described previously (Mailand and Diffley, 2005; Mailand et al., 2006). All siRNA duplexes (purchased from Thermo Fischer Scientific) were transfected at a final concentration of 100 nM using Lipofectamine RNAiMAX (Invitrogen).

Cell culture

Human U2OS osteosarcoma cells and HEK293T embryonic kidney cells were cultured in DME containing 10% fetal bovine serum. U2OS derivative cell lines expressing Myc-tagged USP7 WT or CI in a doxycycline-inducible fashion from pcDNA4/TO-Myc-USP7 vectors were generated and maintained as described previously (Mailand et al., 2007). The U2OS/Myc-Cdh1 cell line has been described previously (Sorensen et al., 2000). Cells were synchronized in mitosis by shaking off rounded cells after treatment with 40 ng/ml nocodazole (Sigma-Aldrich) for 12 h. Other drugs used in this study included: 1 μ g/ml doxycycline (EMD), 2 μ g/ml tetracycline (EMD), 2 mM HU (Sigma-Aldrich), 25 μ g/ml cycloheximide (Sigma-Aldrich), and etoposide (50 μ M for exponential and 100 μ M for mitotic cells; EMD).

Immunocytochemical methods and microscopy

Immunoblotting (IB), IP, and immunofluorescence were performed as described previously (Mailand et al., 2006). Antibodies used in this study included mouse monoclonals to Strep-tag (IBA BioTAGnology) and Mdm2 (sc-965; Santa Cruz Biotechnology, Inc.); rabbit polyclonals to USP7 (BL851; Bethyl Laboratories, Inc.), Claspin (BL-73 [Bethyl Laboratories, Inc.] and Ab3720 [Abcam], used for IB and immunofluorescence, respectively), Wee1 (sc-325; Santa Cruz Biotechnology, Inc.), and USP28 (a gift from S.J. Elledge, Harvard Medical School, Boston, MA). Antibodies to FLAG, Myc, HA, cyclin B1, Chk1, Cdk7, Chk1 S317, SMC1, TopBP1, and MCM6 have been described previously (Mailand et al., 2006). Acquisition of confocal images was done essentially as described previously (Bekker-Jensen et al., 2006), using a confocal microscope (LSM510; Carl Zeiss, Inc.) fitted with a Plan-Neofluar 40x NA 1.3 oil immersion objective lens (Carl Zeiss, Inc.). For dual-color imaging, secondary antibodies coupled to Alexa Fluor dyes with excitation wavelengths of 488 and 568 nm were used. Image acquisition and basic image processing were performed with LSM software (Carl Zeiss, Inc.).

In vitro deubiquitylation assay

Claspin was ubiquitylated in vitro essentially as described previously (Mailand et al., 2006). In brief, in vitro-translated [³⁵S]Claspin (amino acids 1–380) was incubated with ubiquitylation reaction mix (40 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 1 mM DTT, 10% glycerol, 1 mg/ml ubiquitin, 10 mM phosphocreatine, 100 μ g/ml creatine phosphokinase, 0.5 mM ATP, 5 μ M MG132 [all from Sigma-Aldrich], 5 μ M human E1, and 2 μ M hUbcH5C [both from Boston Biochem]), then supplemented with 30 μ g of extract from exponentially growing or mitotic U2OS cells (Mailand et al., 2006) and unlabeled in vitro-translated β TrCP1. To assess the deubiquitylation activity of USP7 toward Claspin, 1 μ g of GST-USP7 WT purified from *Escherichia coli* was included in the reaction. Reactions were incubated at 30°C for 1 h, stopped by addition of Laemmli sample buffer, resolved by SDS-PAGE, and visualized by autoradiography.

Online supplemental material

Fig. S1 shows specificity and effects of USP7, Claspin, and Chk1 siRNAs. Fig. S2 shows characterization of the U2OS/Myc-USP7 inducible cell lines. Fig. S3 shows cell cycle profiles of cells in the experiment in Fig. 4 B at representative time points, determined by flow cytometric analysis. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200807137/DC1>.

We thank Roger Everett and Stephen J. Elledge for providing reagents, and Michele Pagano for communicating unpublished results.

This work was supported by grants from the Danish Cancer Society (DP06009), Danish National Research Foundation, Danish Medical Research Council (271-07-0047), European Commission (integrated projects "DNA Repair" [2005-512113] and "Active p53" [2004-503576]), and the John and Birthe Meyer Foundation.

Submitted: 23 July 2008

Accepted: 11 December 2008

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Deubiquitylation of Claspin by USP7

Claspin and USP7

The checkpoint mediator protein Claspin, that facilitates ATR-mediated phosphorylation and activation of Chk1, was found to interact with the deubiquitylating enzyme USP7. The two proteins interacted *in vivo* and over-expression of USP7 was found to stabilize Claspin. Furthermore, over-expression of USP7 increased the half-life of Claspin and deubiquitylated Claspin *in vivo* and *in vitro*. USP7 was therefore suggested to be a novel and positive regulator of Claspin stability. Upon entry into mitosis and upon recovery from DNA damage induced cell cycle arrest, Claspin is ubiquitylated by SCF^{βTrCP} leading to its proteasomal degradation (Mailand et al., 2006; Mamely et al., 2006; Peschiaroli et al., 2006). Claspin was stabilized in mitotic cells upon over-expression of USP7, which was suggested to oppose the degradation of Claspin by deubiquitylation and stabilization. In released mitotic cells over-expressing USP7, Claspin was degraded upon entry into G1 and USP7 was therefore suggested not to stabilize Claspin in G1.

In G1, Claspin level was found to be regulated by APC^{Cdh1} instead of SCF^{βTrCP} which is also observed by Bassermann et al., 2008. The additional requirement of APC to remove Claspin during G1 might be explained by the need for the priming kinase Plk1, and since Plk1 is absent in G1 due to destruction by APC^{Cdh1} (Lindon and Pines, 2004; Peters, 2006), it necessitate a Plk1 independent mechanism for degradation of Claspin in G1. APC^{Cdh1} maintains the G0 and G1 state by ensuring that many positive regulators of S and M do not accumulate prematurely. Claspin was previously suggested to have a positive role in replication since over-expression of Claspin has been shown to increase cell proliferation (Lin et al., 2004) and Claspin is therefore suggested to be kept at a low level to avoid premature entry into S (Bassermann et al., 2008). Still, it seems unlikely that Claspin should have both a negative and positive role on cell cycle progression. Furthermore, unscheduled accumulation of Claspin in G1 is not desired and could perhaps stabilize and activate of Chk1 in the absence of DNA damage.

Another substrate that is also regulated by both SCF^{βTrCP} upon checkpoint activation and entry into mitosis and APC^{Cdh1} in early G1 is Cdc25A (Donzelli et al., 2002). Cdc25A degradation as well as Cyclin A and Cyclin B allow inactivation of Cdk1 upon mitotic exit, the action of which is not needed after mitosis and is detrimental in G1 due to the unidirectionality of the cell cycle. This furthermore helps the cells to reset before Cdc25A starts to accumulate at the next G1/S transition (Busino et al., 2004). Analogous to Claspin, Cdc25A has a normal use in the cell cycle and is kept low in G1, but have more sophisticated regulation of the activity in S and G2.

USP7 only seems to regulate Claspin levels in G2/M and not in G1. It is suggested that a tight regulation of Claspin in G2/M is more important compared to G1, where Claspin stabilization is not desirable. This raises the questions if ubiquitin chains produced by SCF^{βTrCP} are more accessible to USP7 than ubiquitin chains produced by APC^{Cdh1}, and if the activity of USP7 is cell cycle regulated. It is more likely that the regulation is at the substrate level since USP7 was shown to have an effect on Mdm2 stabilization in G1. Furthermore, the levels of endogenous USP7 were analyzed in exponentially growing cells, in cells treated with HU for 24 hours and in mitotic shaken off cells, but no change in the levels of USP7 were observed (unpublished data).

It would therefore be interesting to investigate if the SCF^{βTrCP}-mediated ubiquitin chains are differently susceptible for USP7 deubiquitylation than APC^{Cdh1}-mediated ubiquitin chains. There are indications that DUBs may act on different ubiquitin chains (Nijman et al., 2005b). Claspin ubiquitin chains assembled by SCF are very likely K48, which is the most common ubiquitin linkage applied

to target proteins for degradation. It was also previously shown that SCF together with its E2 Cdc34 facilitate K48 chain formation (Gazdoui et al., 2005; Petroski and Deshaies, 2005). But the exact composition of ubiquitylated Claspin has not been investigated so far. Recently, human APC together with E2 UbcH10 was shown to target proteins for degradation by preferentially assembling K11 ubiquitin chains (Jin et al., 2008). It could therefore be interesting to analyze if SCF- and APC-mediated ubiquitin chains on Claspin have different composition.

Upon DNA damage encountered in G2, the phosphatase Cdc14B is suggested to translocate from the nucleolus to the nucleoplasm and induces activation of APC^{Cdh1}, which degrades Plk1. Plk1 degradation stabilizes Claspin and Wee1 to allow efficient G2/M checkpoint. Since Claspin is also a target of APC^{Cdh1} in G1, USP28 is suggested to protect Claspin from APC^{Cdh1} mediated degradation in response to DNA damage (Bassermann et al., 2008). The data presented here suggest that USP7 has a role in maintaining the steady-state level of Claspin. It could therefore be interesting to investigate the USP7- and USP28-mediated deubiquitylation and stabilization of Claspin in more details. Is Claspin ubiquitylated with ubiquitin chains of different composition, which could then be targeted differently by the two DUBs, and how is this regulated?

Down-regulation of USP7 prevented activation of Chk1 upon exposure to UV, whereas over-expression interfered with the cells ability to timely degrade Claspin and inactivate Chk1 upon checkpoint termination. These data suggest that a tight coordination of Claspin ubiquitylation and deubiquitylation is needed to enforce Claspin degradation and thus Chk1 inactivation in mitosis. It could therefore be interesting to investigate if USP7 may have a direct role in initiation as well as termination of Chk1-mediated signaling responses.

The results obtained contribute to a better understanding of the dynamic and complex regulation of Claspin stability and its importance during cell cycle and upon checkpoint inducing stimuli. Furthermore it highlights the importance of the opposing ubiquitylation and deubiquitylation activities in regulating protein levels and on timing cell cycle coordination after DNA damage.

5 Abbreviations

9-1-1	Rad9-Rad1-Hus1
19S	Subunit of the 26S proteasome
20S	Subunit of the 26S proteasome
26S	Proteasome
53BP1	p53 Binding Protein 1
APC	Anaphase promoting complex (E3 ubiquitin ligase complex)
ATM	Ataxia-telangiectasia mutated
ATR	Ataxia-telangiectasia and Rad3 related
ATRIP	ATR interacting protein
BACH1	BRCA1-associated C-terminal helicase
BRCA1	Breast cancer susceptibility gene 1
BRCT	BRCA1 C-terminal (protein domain)
βTrCP	Beta-Transducin repeat containing protein (F-box protein for SCF complex)
Cdc25	Cell division cycle 25 (family of dual specificity phosphatases)
Cdc20	Cell division cycle 20 (activator protein for APC complex)
Cdc45	Cell division cycle 45 (An essential replication protein)
Cdh1	Cadherin 1 (activator protein for APC complex)
Cdk	Cyclin dependent kinase
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
CKI	Cdk inhibitor
CtIP	C-terminal binding protein-interacting protein
DDB2	DNA damage binding protein 2
DDR	DNA damage response
DSB	Double-strand break
DUB	Deubiquitylating enzyme
Emi1	Early mitotic inhibitor 1
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligating enzyme
FANCD2	Fanconi anemia complementation group D2
FANCL	Fanconi anemia complementation group L (Ubiquitin ligase activity)
Fbw7	F-box and WD repeat domain-containing 7 (F-box protein for SCF complex)
γ-H2AX	Phosphorylated H2AX
G0	Quiescent stage (non-replicative stage)
G1 & G2	Gap phase 1 and gap phase 2 of the cell cycle
GST	Glutathione-S-transferase (Epitope tag)
H2A	Histone 2A
H2B	Histone 2B
H2AX	Histone 2A variant
H3	Histone 3
H4	Histone 4
HECT	Homologous to E6-AP carboxyl terminus (domain found in many E3s)
HR	Homologous recombination
HU	Hydroxy Urea
IR	Ionizing radiation
IRIF	Ionizing radiation induced foci

Abbreviations

kDa	Kilodalton
MDa	Megadalton
Mdc1	Mediator of DNA damage checkpoint 1
Mdm2	Mouse double minute 2 homolog (p53 binding protein, E3 ligase)
MIU	Motif interacting with ubiquitin (Ubiquitin binding domain)
MRN	Mre11-Rad50-Nbs1 complex
NBS1	Nijmegen breakage syndrome 1
NHEJ	Non-homologous end joining
PCNA	Proliferating cell nuclear antigen
PHD	Plant homeo domain (a class of E3 ubiquitin ligases)
Plk1	Polo-like kinase 1
p21	Cyclin-dependent kinase inhibitor
p27	Cyclin-dependent kinase inhibitor
p53	Transcription factor
p57	Cyclin-dependent kinase inhibitor
R	Restriction Point
Rb	Retinoblastoma protein
Rad6	Identified in radiation sensitive mutant (Ubiquitin conjugating enzyme)
Rad17	Identified in radiation sensitive mutant (Checkpoint protein)
Rad18	Identified in radiation sensitive mutant (Ubiquitin ligase)
Rad51	Identified in radiation sensitive mutant (repair protein)
Rap80	Receptor associated protein 80
RING	Really interesting new gene. RING-finger found in many E3 ubiquitin ligases
RNF8	RING domain nuclear factor 8
ROS	Reactive oxygen species
RPA	Replication protein A
SCF	Skp1-Cul1-F-box protein complex (E3 ubiquitin ligase complex)
Skp2	S-phase kinase associated protein 2 (F-box protein for SCF complex)
SSB	Single-strand break
ssDNA	Single-stranded DNA
TLS	Translesion synthesis
TopBP1	Topoisomerase DNA II binding protein 1
U-box	Domain found in the U-box family of E3 ubiquitin ligases
Ubc13	Ubiquitin conjugating enzyme 13
UBD	Ubiquitin binding domains
UIM	Ubiquitin-interacting motif
USP	Ubiquitin specific protease
UV	Ultra violet (light)
Wee1	A dual specificity protein kinase
XPC	Xeroderma Pigmentosum complementation group C

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7 Appendix Publication 1

Cell, Volume 131

Supplemental Data

RNF8 Ubiquitylates Histones at

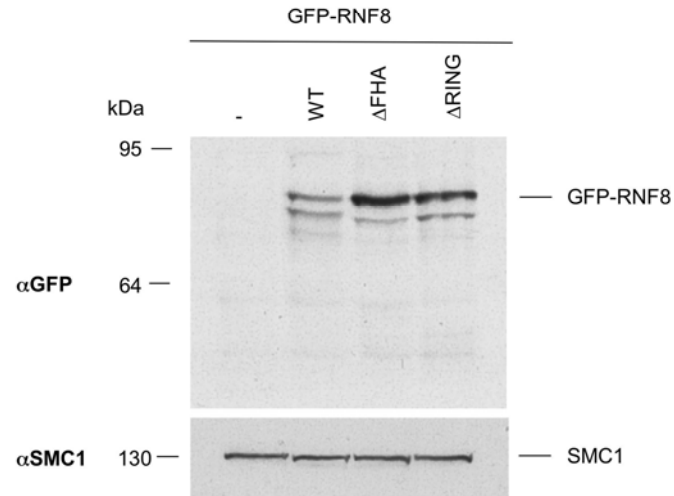
DNA Double-Strand Breaks and

Promotes Assembly of Repair Proteins

Niels Mailand, Simon Bekker-Jensen, Helene Faustrup, Fredrik Melander, Jiri Bartek,
Claudia Lukas, and Jiri Lukas

Figure S1 (A, B)

A



B

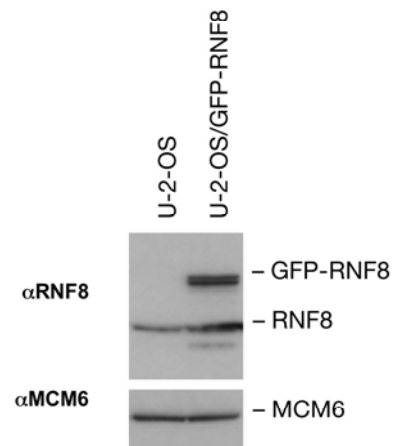


Figure S1 (C)

C

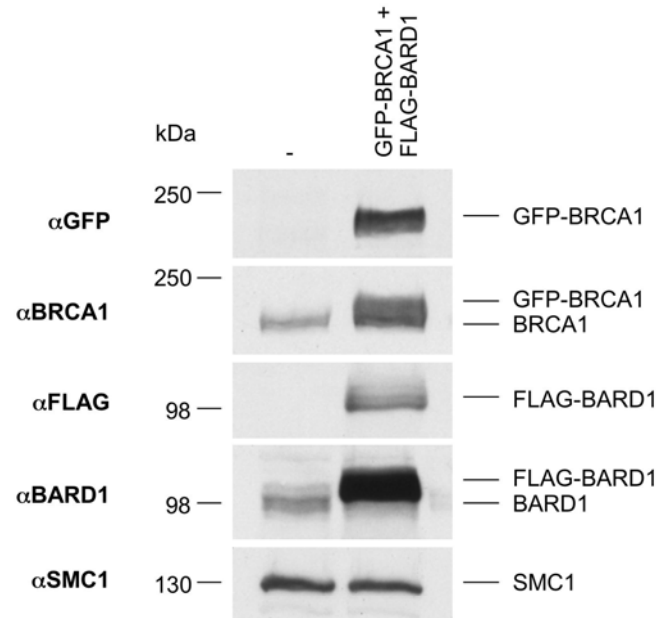
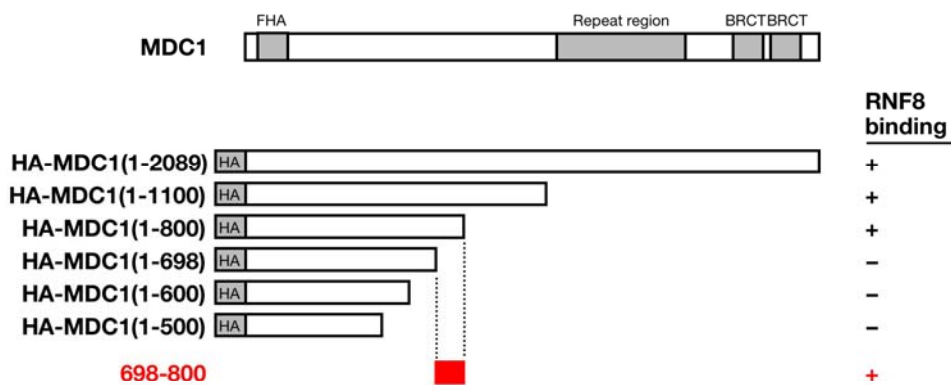


Figure S1. Characterization of new cell lines expressing GFP-tagged DSB regulators

- (A) Lysates from U-2-OS cells stably expressing the indicated forms of GFP-RNF8 were analysed by immunoblotting with an anti-GFP antibody. SMC1 served as a loading control.
- (B) Lysates from U-2-OS cells stably expressing GFP-RNF8 were analysed by immunoblotting with a rabbit antibody to RNF8. GFP-RNF8 was expressed at levels comparable to the endogenous proteins. MCM6 served as a loading control.
- (C) U-2-OS cells stably co-expressing GFP-BRCA1 and FLAG-BARD1 were analysed by immunoblotting with the indicated antibodies for migration and abundance of the integrated transgenes. SMC1 is a loading control.

Figure S2

A



B

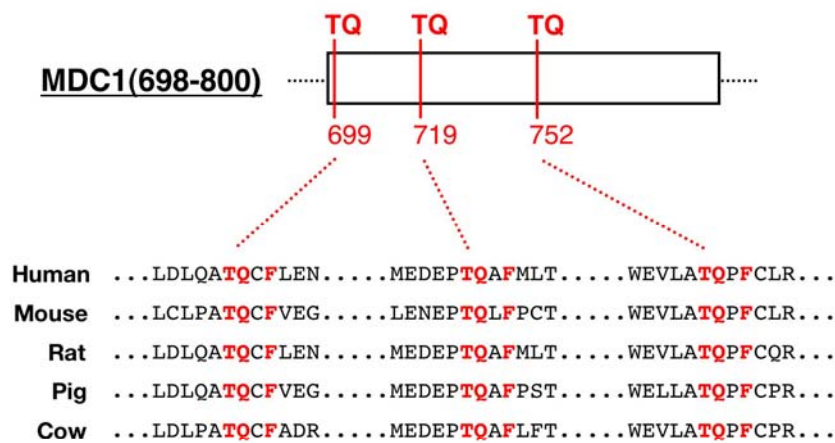
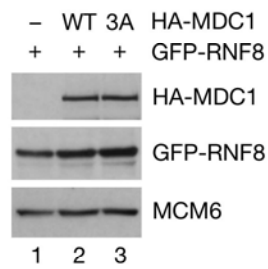


Figure S2. Structural requirements of MDC1 for RNF8 interaction

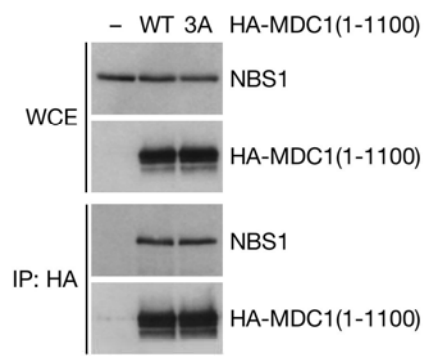
- (A) The indicated fragments of HA-tagged human MDC1 were co-expressed with FLAG-RNF8 in 293T cells, and their interaction was measured by immunoprecipitation with anti-HA-antibody followed by immunoblotting with anti-FLAG antibody (the same conditions as in Fig. 2B). The drawing schematically illustrates the interaction capabilities of the respective MDC1 fragments, and indicates the minimal region of MDC1 (red) that is necessary and sufficient to interact with RNF8.
- (B) A graphical alignment of conserved T-Q-X-F residues in MDC1 proteins from different mammalian species.

Figure S3 (A-C)

A



B



C

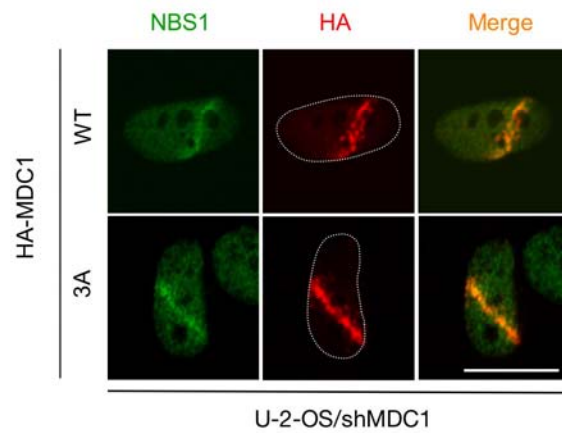
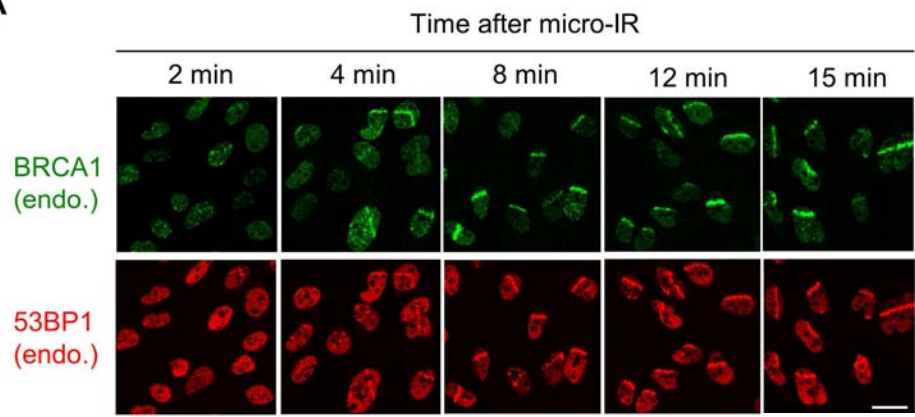


Figure S3. Disruption of the conserved T-Q-X-F domain in the MDC1 terminus does not affect NBS1 chromatin retention

- (A) U-2-OS/shMDC1 cells were co-transfected with GFP-RNF8 and the indicated versions of HA-MDC1 and assayed by immunoblotting with the indicated antibodies. MCM6 is a loading control.
- (B) U-2-OS cells were transfected with the wild-type (wt) HA-MDC1 or its variant where the threonines within all three conserved T-Q-X-F repeats were substituted by alanines (3A). Association between HA-MDC1 and endogenous NBS1 was analyzed by immunoprecipitation with anti-HA antibody followed by immunoblotting with NBS1 antibody.
- (C) U-2-OS/shMDC1 cells were transfected with the two forms of HA-tagged MDC1 as in (A), miroirradiated by the laser, and co-immunostained with antibodies to HA (to confirm productive expression of the MDC1 proteins and their ability to localize to DSB sites) and NBS1. WCE, whole cell extract.
- Scale bars, 10 μ m.

Figure S4 (A, B)

A



B

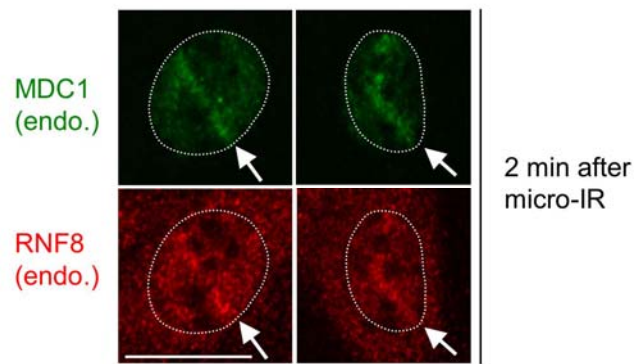


Figure S4(C)

C

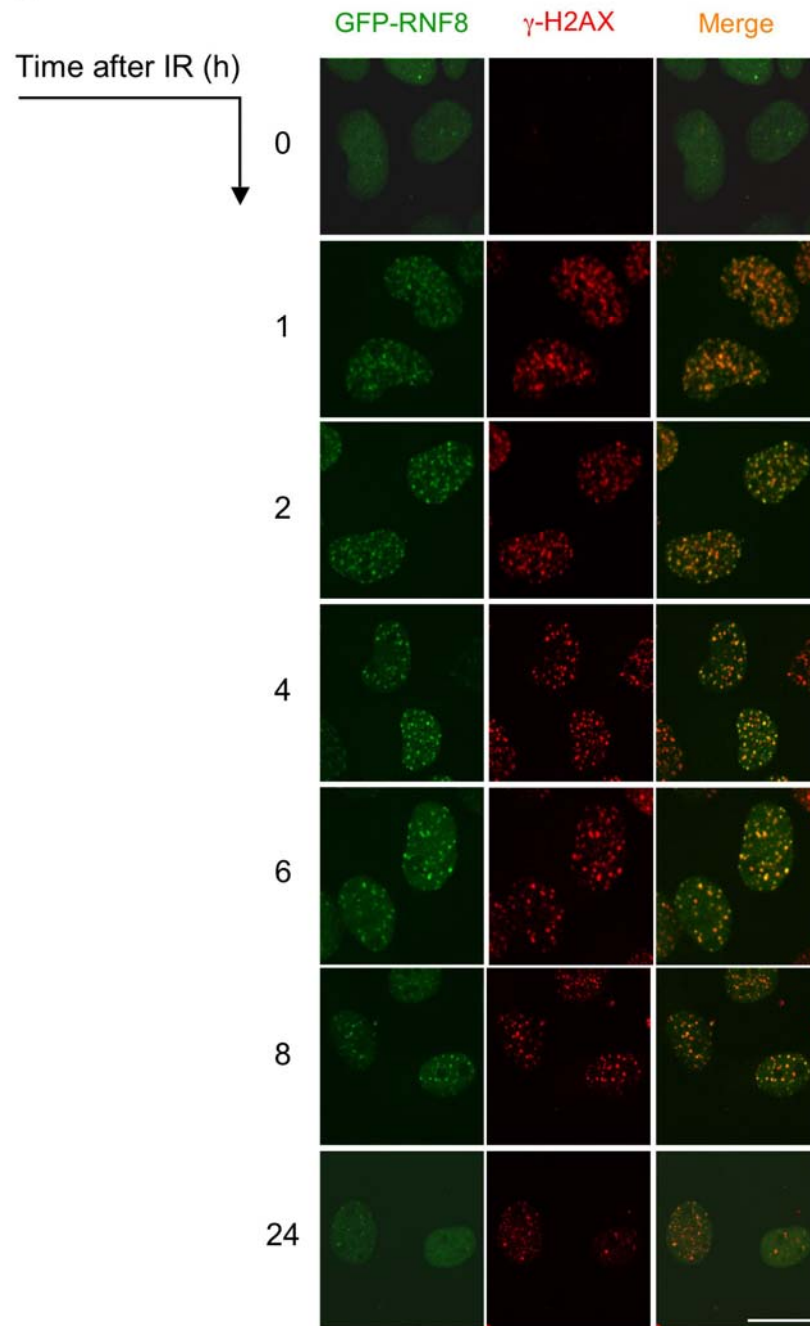


Figure S4. Endogenous BRCA1 and 53BP1 assemble at the DSB sites with a similar kinetics

- (A) U-2-OS cells were exposed to laser microirradiation, and at the indicated times fixed and co-immunostained with antibodies to endogenous BRCA1 and 53BP1. The appearance and magnitude of both proteins in the microirradiated areas (and within the same cells) parallel each other. Scale bar, 10 μ m.
- (B) U-2-OS cells were microirradiated as in A and co-immunostained with antibodies to RNF8 and MDC1. Recruitment of endogenous RNF8 was evident from the earliest time points (2 min after microirradiation) when accumulation Mdc1 became clearly detectable at the laser-generated DSB tracks.
- (C) U-2-OS cells stably expressing physiological levels of GFP-RNF8 (see Fig. S1B) were exposed to IR (4 Gy) and at the indicated time-points immunostained with an antibody to γ -H2AX. During the entire time-course, the localization and intensity of GFP-RNF8 closely paralleled that of the γ -H2AX-decorated chromatin. Scale bars, 10 μ m.

Figure S5

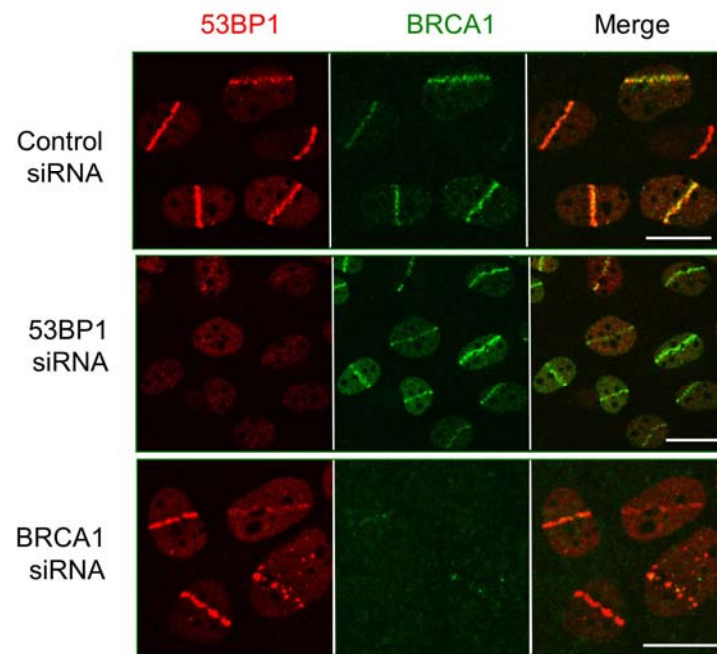


Figure S5. Accumulation of 53BP1 and BRCA1 at the DSB sites is independent of each other

U-2-OS cells were treated with the indicated siRNA oligonucleotides for 72 h, subjected to laser microirradiation, and after additional 30 min fixed and co-immunostained with the indicated antibodies. siRNA oligonucleotides to 53BP1 (Bekker-Jensen et al., 2005), and GL2 (control; Mailand et al., 2006) were described. siRNA to BRCA1 was a SmartPool (MU-003461-01; Dharmacon) containing four oligonucleotides to the following sequences (sense strand): 5'-CAGCUACCCUCCAUCAUAUU-3'; 5'-GGGAUACCAUGCAACAUAUU-3'; 5'-GAAGGAGCUUCAUCAUUCUU-3'; 5'-CUAGAAAUCUGUUGCUAUGUU-3'. Scale bars, 10 μ m.

Figure S6 (A)

A

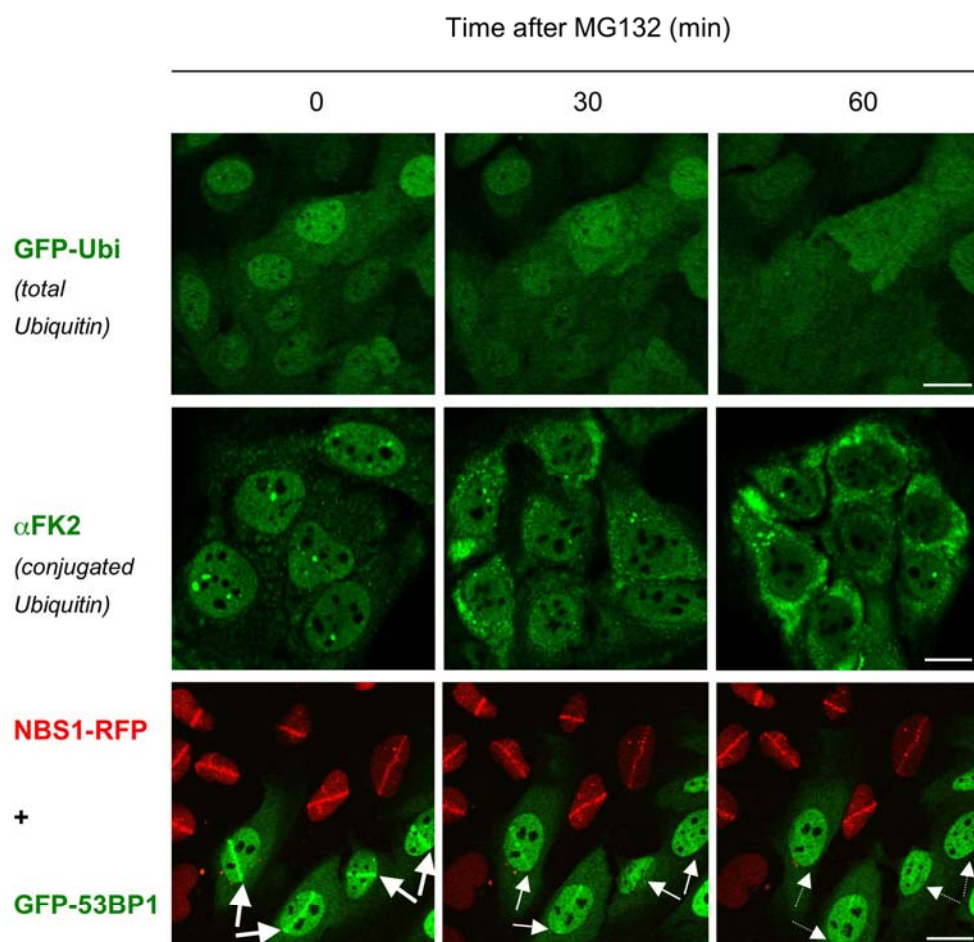


Figure S6 (B)

B

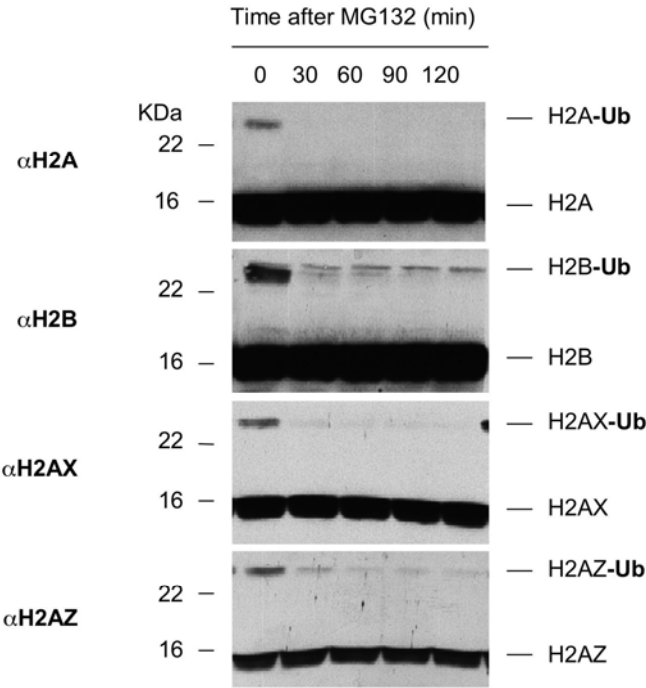
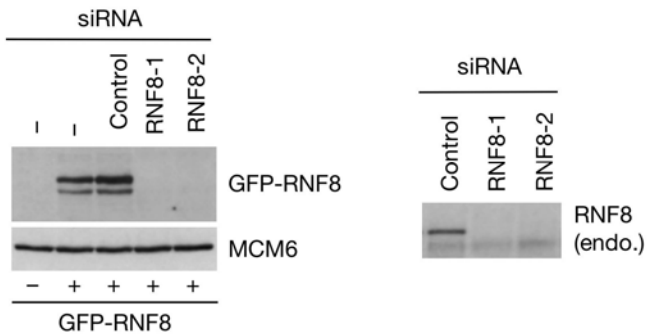


Figure S6. Inhibition of the 26S proteasome impairs regulatory ubiquitylations in the cell nucleus and inhibits 53BP1 retention in the DSB compartment

- (A) U-2-OS cells stably expressing GFP-tagged ubiquitin were treated with the MG132 proteasome inhibitor (5 μ M) and subjected to time-lapse microscopy. The GFP fluorescence within the same field was recorded at the indicated time-points (top). U-2-OS cells were treated as above and immunostained with an FK2 antibody to conjugated ubiquitin (middle). U-2-OS cells stably expressing NBS1-RFP and GFP-53BP1 respectively, were co-cultivated in the same imaging chamber, microirradiated, and incubated for 30 min to allow assembly of both proteins at the DSB-flanking chromatin. Afterwards, MG132 was added to the culture medium, the cells were subjected to live cell time-lapse microscopy, and images were captured at indicated time-points (bottom). Note that the dissociation kinetics of 53BP1 (but not NBS1) after MG132 addition coincides with the depletion of nuclear ubiquitin. Scale bars, 10 μ m.
- (B) U-2-OS cells were treated with MG132 as in (A). At the indicated time-points, the cell lysates were acid extracted to enrich for nucleosomal histones as described (<http://www.abcam.com/assets/pdf/protocols/Histone%20extraction%20protocol.pdf>) and analysed by immunoblotting with the indicated antibodies (left). Rabbit polyclonal antibodies used in this experiment include: H2A (Abcam; ab18255), H2B (Abcam; ab1790), H2AX (Cell Signalling, 2595), and H2AZ (Upstate, 07-594).

Figure S7 (A, B)

A



B

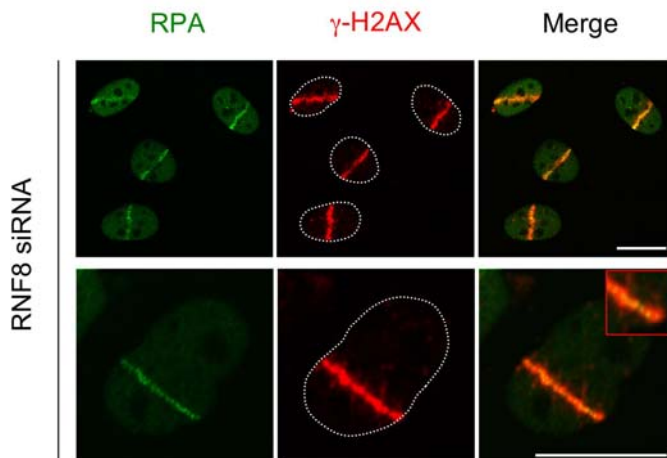
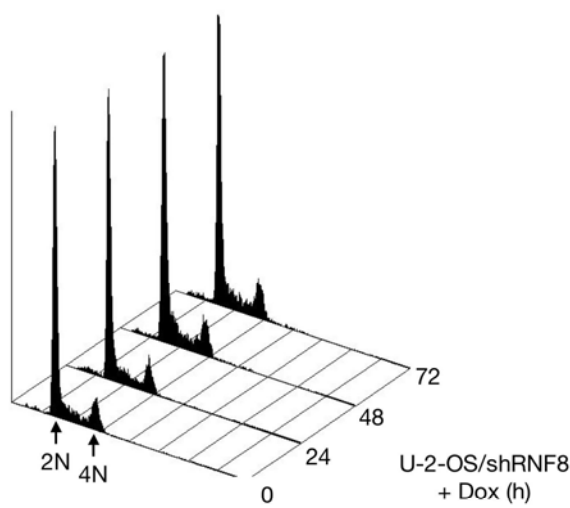


Figure S7 (C, D)

C



D

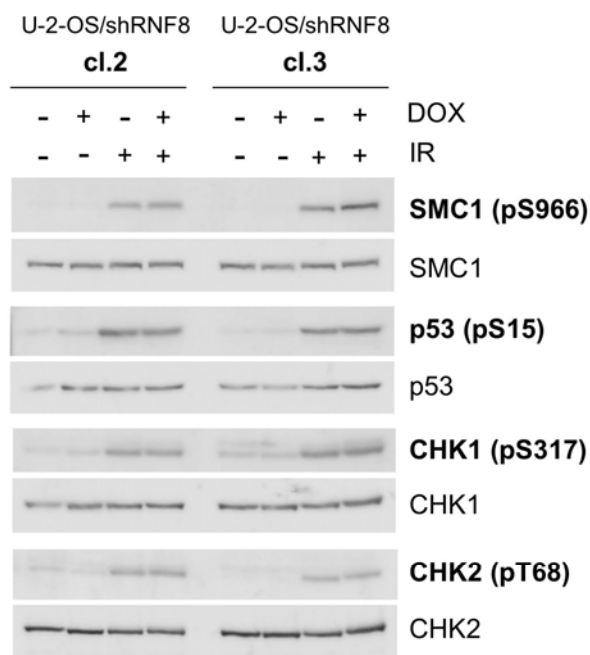


Figure S7. Effects of RNF8 knockdown on DSB resection, nuclear mobility of 53BP1, cell cycle progression, and DNA damage signalling

- (A) U-2-OS cells were transfected, or not with GFP-RNF8. Twenty-four h later, cells were co-transfected with indicated siRNAs for an additional 48 h, and the efficiency of the siRNAs was monitored by immunoblotting with anti-GFP antibody. MCM6 served as a loading control (left). U-2-OS cells were transfected with the indicated siRNAs for 48 h. Levels of the endogenous RNF8 protein were determined by immunoprecipitation of RNF8 with goat anti-RNF8 antibody (Ab-15850, AbCam), followed by immunoblotting with rabbit anti-RNF8 antibody (Ab-4183, AbCam) (right).
- (B) U-2-OS cells were treated with RNF8-targeting siRNA oligonucleotides for 48 h, subjected to laser microirradiation, and 30 min later immunostained with the indicated antibodies. Accumulation of RPA in characteristic sub-chromatin ‘microfoci’ within the microirradiated tracks (inset) indicates that the DSB resection and generation of single stranded DNA was not impaired by RNF8 depletion. Mouse monoclonal antibody to the p34 subunit of RPA (Ab-1) was obtained from Lab Vision. Scale bars, 10 μ m.
- (C) U-2-OS cells conditionally expressing RNF8-targeting shRNA (U-2-OS/shRNF8) were induced by Doxycycline (Dox). At the indicated time points, the cell cycle distribution was analyzed by flow cytometry as described (Mailand et al., 2006).
- (D) Two different clones (cl. 2 and cl. 3) of U-2-OS/shRNF8 cells were induced, or not by Dox, and subjected (or not) to IR (3 Gy). One hour later, the cell lysates were analysed by immunoblotting with antibodies to total or phosphorylated forms of the indicated proteins. Antibodies in this experiment

include: SMC1 (rabbit; Abcam), SMC1-pS966 (rabbit; Abcam), p53 (mouse; DO-1), p53-pS15 (rabbit; Cell Signaling), Chk1 (mouse; DCS-316), Chk1-pS317 (rabbit; Cell Signaling), Chk2 (mouse; DCS-270), Chk2-pT68 (rabbit; Cell Signaling).

Figure S8

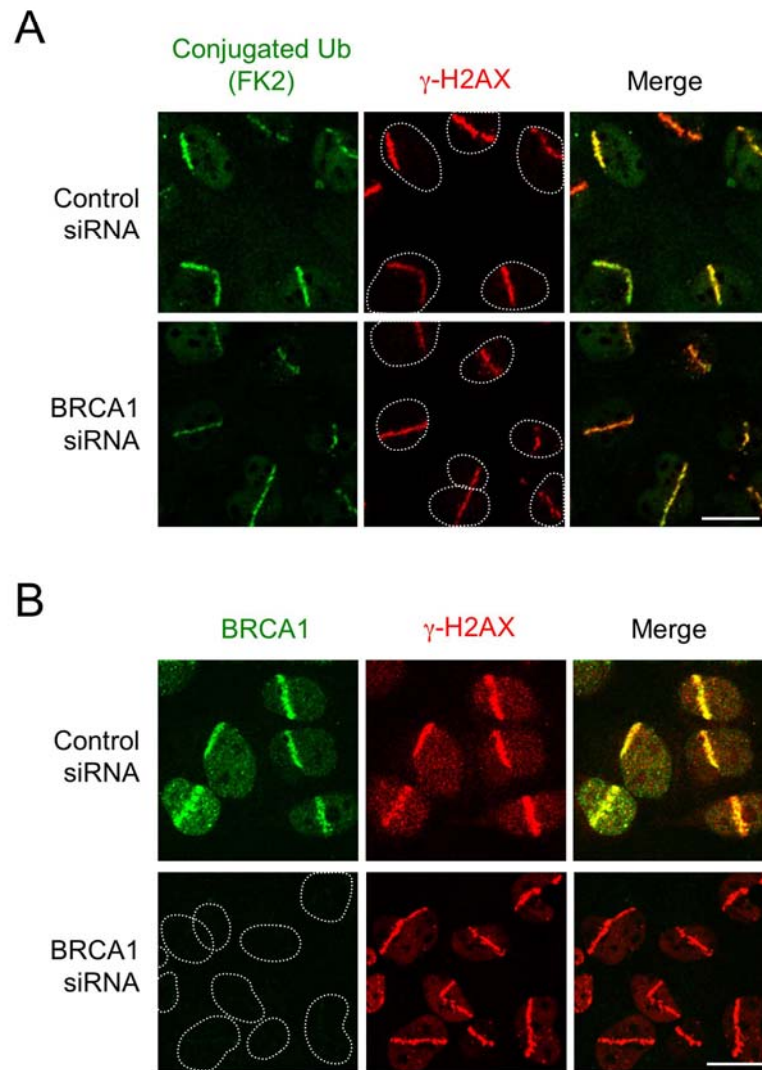
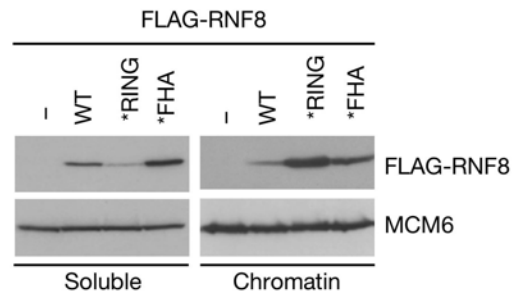


Figure S8. Effect of BRCA1 knockdown on accumulation of conjugated ubiquitin in the microlaser-generated DSB tracks

- (A) U-2-OS cells were treated with the indicated siRNA oligonucleotides for 72h, exposed to laser microirradiation, and after additional hour co-immunostained with antibodies to γ -H2AX and conjugated ubiquitin (FK2). The conditions in this experiment were identical to those applied in Figures 4B. The siRNA sequences for BRCA1 were as in Fig. S5.
- (B) U-2-OS cells were treated with siRNA oligonucleotides as in (A) and co-immunostained with antibodies to BRCA1 and γ -H2AX. Note that the siRNA-mediated downregulation of BRCA1 was very efficient (under these conditions, both the nucleoplasmic and the DSB-associated fluorescent signal of BRCA1 was reduced below the detection level). Scale bars, 10 μ m.

Figure S9 (A, B)

A



B

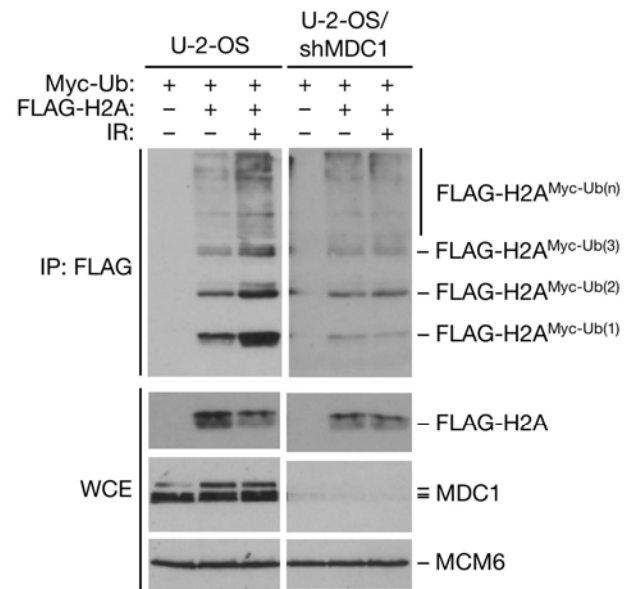
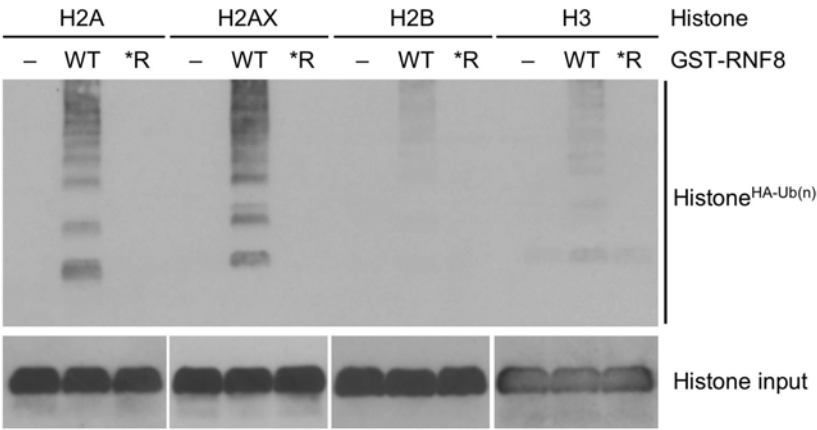
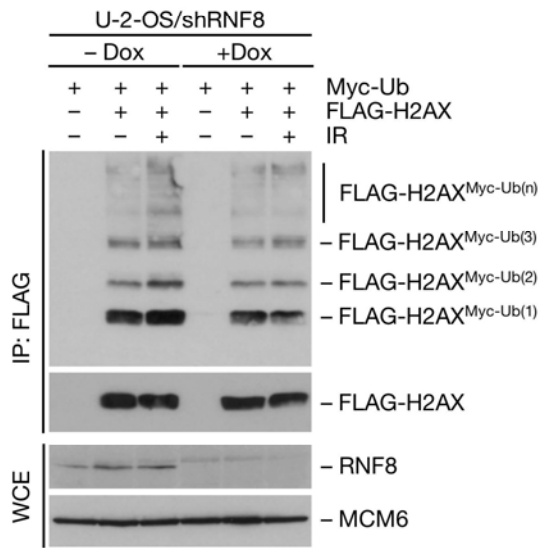


Figure S9 (C-E)

C



D



E

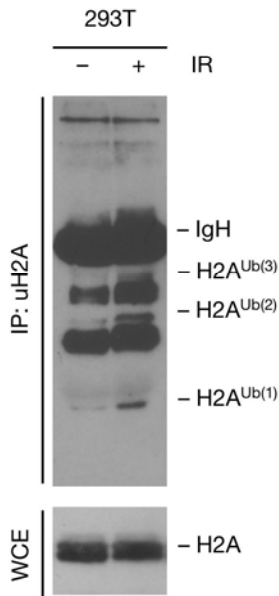


Figure S9. Enhanced interaction of the catalytically inactive RNF8 with chromatin, and the MDC1 requirement for the IR-induced H2A ubiquitylation

- (A) U-2-OS cells were transfected with indicated FLAG-RNF8 constructs for 24 h. Subsequently, cells were fractionated to separate soluble and chromatin-enriched proteins as described (Mailand et al., 2006), and the resulting extracts were analyzed by immunoblotting.
- (B) U-2-OS cells or their derivatives stable expressing the MDC1-targetting shRNA (U-2-OS/shMDC1) were transfected with the indicated constructs and the H2A ubiquitylation was monitored as in Fig. 6C.
- (C) In vitro ubiquitylation of histones H2A, H2AX, H2B, and H3 by RNF8 was analyzed as in Fig. 6D. The degree of histone ubiquitylation was monitored by immunoblotting with anti-HA antibody on the same gel to directly compare the ability of RNF8 to ubiquitylate individual histones (upper panel). The blot was then stripped and reprobed with antibodies to individual histones (lower panels).
- (D) In vivo ubiquitylation of H2AX. U-2-OS/shRNF8 cells were left untreated or induced by addition of Dox. Twenty-four h later, cells were transfected with the indicated constructs, and the ubiquitylation of H2AX was monitored as in Fig. 6C.
- (E) HEK293T cells were mock-treated or exposed to IR (20 Gy) for 1 h. Cells were then lysed in EBC buffer containing 500 mM NaCl and sonicated, and extracts were subjected to immunoprecipitation with anti-ubiquityl-H2A antibody (uH2A). Bound complexes were resolved by SDS-PAGE and analyzed by immunoblotting with Histone H2A antibody. IgH, immunoglobulin heavy chains.

8 Appendix publication 2

Faustrop et al., <http://www.jcb.org/cgi/content/full/jcb.200807137/DC1>

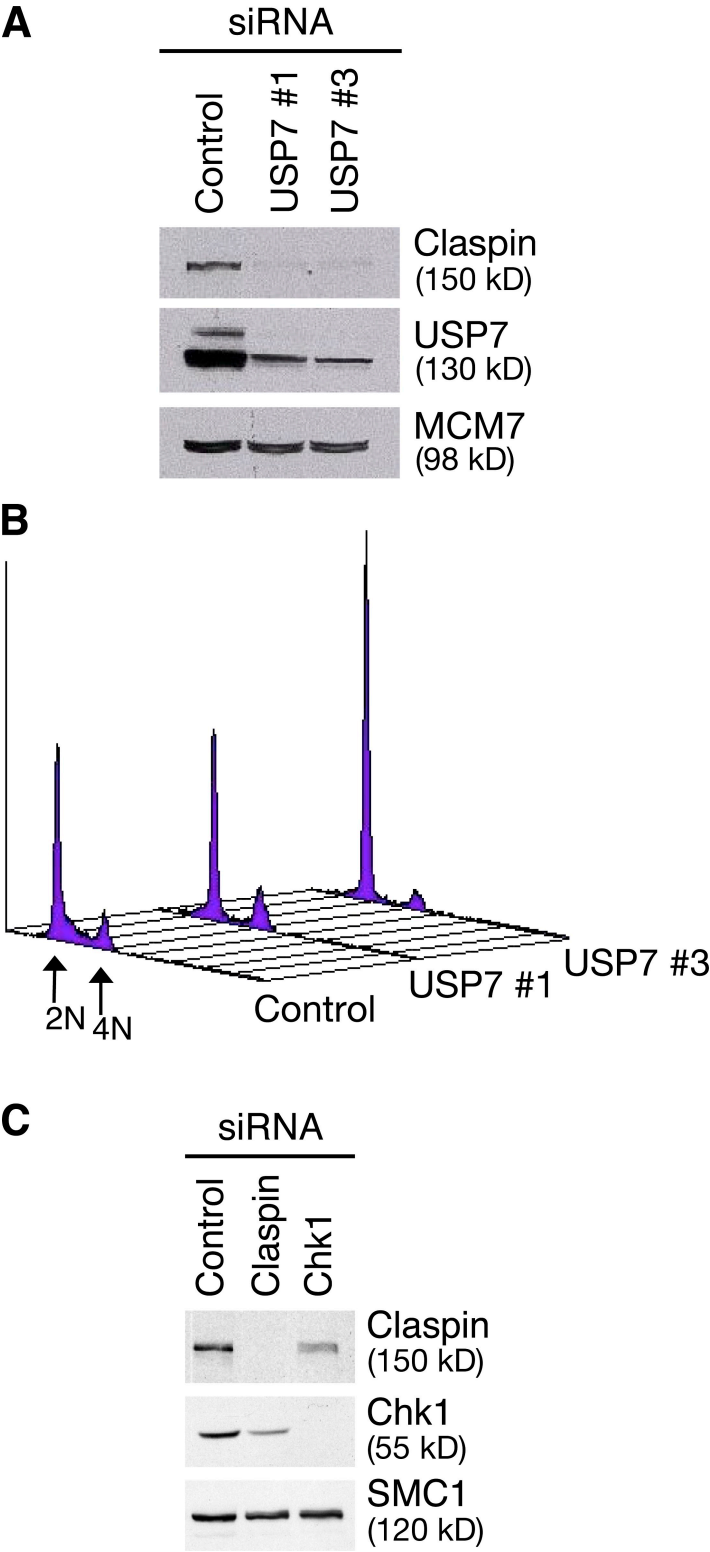


Figure S1. **Effects of USP7, Claspin, and Chk1 siRNAs.** (A and B) siRNA-mediated depletion of USP7 down-regulates Claspin levels. U2OS cells were transfected with control or USP7 siRNAs for 72 h, and total cell extracts were processed for IB of indicated proteins (A) or for flow cytometric analysis of cell cycle distribution (B). (C) Claspin and Chk1 promote the stability of each other. U2OS cells were transfected with indicated siRNAs for 72 h, and the expression levels of Chk1 and Claspin were monitored by IB. SMC1 served as a loading control. siRNA sequences used were: USP7 No. 1 (5'-GGCAACCUUUCAGUUCACUTT-3'), USP7 No. 3 (5'-GGCGAAGUUUUAAAUGUAUTT-3'), and control (Hsp70, 5'-GGGAGGACAAGACGUUCUATT-3'). The Chk1 siRNA has been described previously [Syljuasen, R.G., C.S. Sorensen, L.T. Hansen, K. Fugger, C. Lundin, F. Johansson, T. Helleday, M. Sehested, J. Lukas, and J. Bartek. 2005. *Mol. Cell. Biol.* 25:3553-3562].

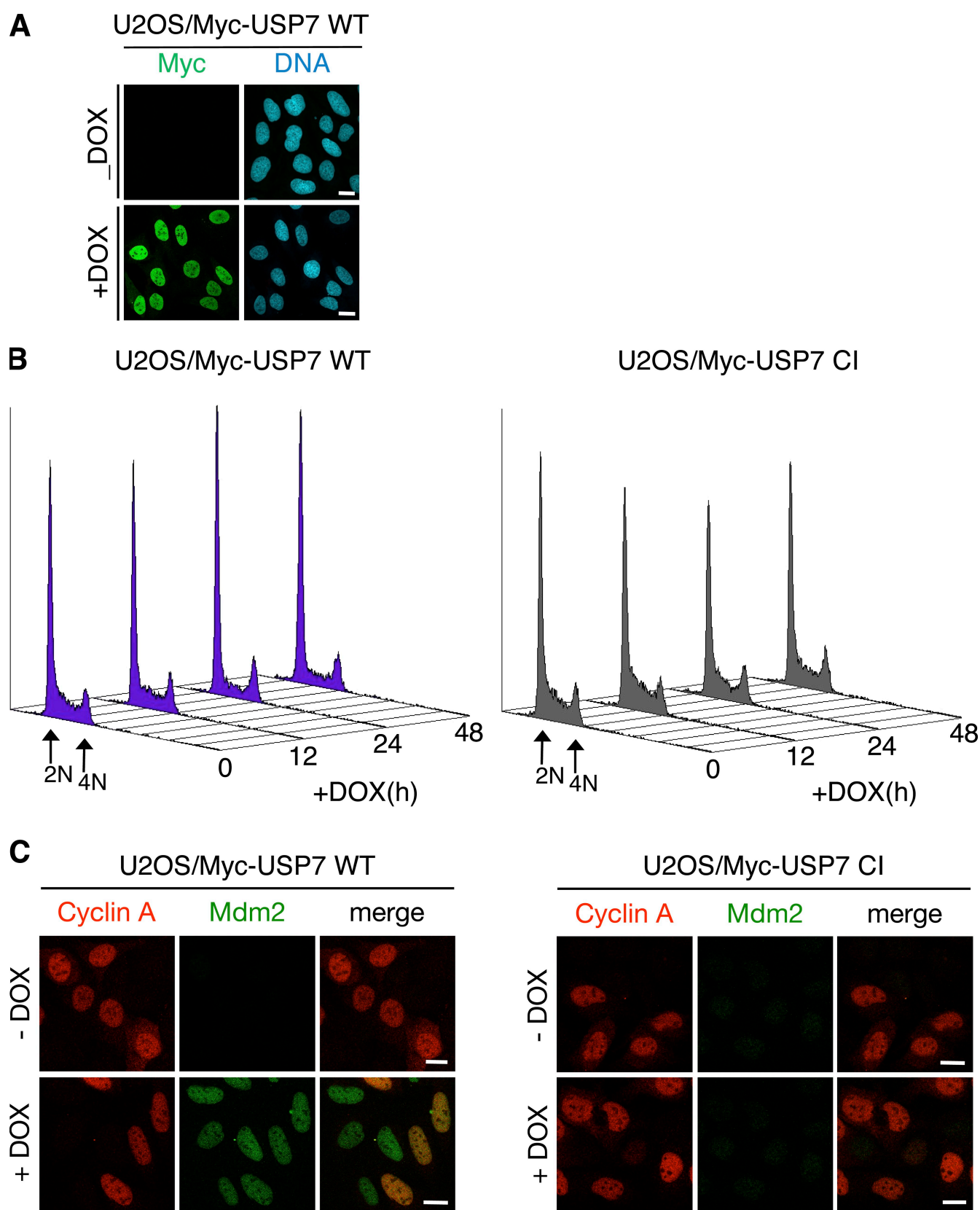


Figure S2. **Characterization of U2OS/Myc-USP7 cell lines.** (A) Conditional U2OS/Myc-USP7 WT cells were left untreated or induced to express the transgene by addition of DOX for 24 h. The cells were then fixed and immunostained with Myc antibody. Bars, 10 μ m. (B) U2OS/Myc-USP7 cell lines were induced with DOX for the indicated times, stained with propidium iodide, and subjected to flow cytometry analysis as described previously (Maidland, N., J. Falck, C. Lukas, R.G. Syljuasen, M. Welcker, J. Bartek, and J. Lukas. 2000. *Science*. 288:1425–1429). (C) Stabilization of Mdm2 in U2OS/Myc-USP7 cells is dependent on the catalytic activity of USP7 but independent of cell cycle stage. U2OS/Myc-USP7 cell lines were left untreated or induced to express the transgenes by the addition of DOX for 24 h. The cells were then fixed and immunostained with Mdm2 and Cyclin A antibodies. Bars, 10 μ m.

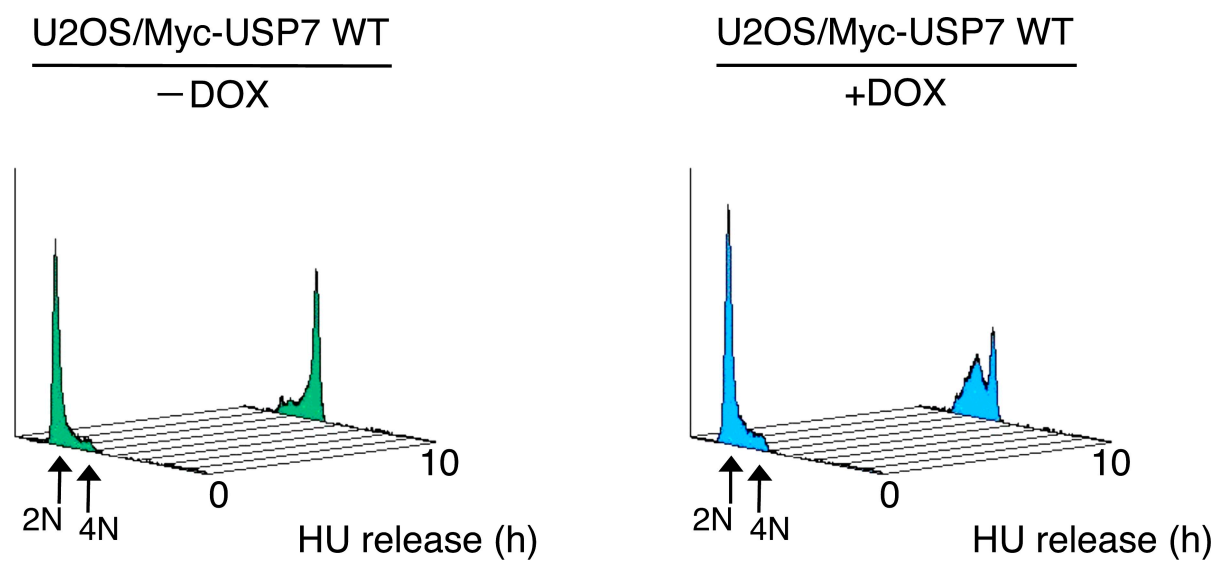


Figure S3. **Representative DNA profiles of the HU release experiment in Fig. 4 B.** U2OS/Myc-USP7 WT cells treated as in Fig. 4 B were processed for flow cytometric analysis of DNA content.